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The effect of BLX-1002 on AMPK and PI3K

Masters Thesis

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Aim of project

- BLX-1002 is a candidate drug for treatment of diabetes that stimulates insulin secretion
- Mechanisms are unknown

- How does BLX-1002 affect the activity of AMPK and PI3K?
  - Impairment of these signalling enzymes may involved in diabetic pathogenesis

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Introduction

- Diabetes -

- World-wide disease that effects over 100 million people
- Two types:
  - Type 1: insulin secretion is impaired or non-existing
  - Type 2: Decreased sensitivity to insulin
    - caused by a shortage of insulin receptors and/or decreased activity of the insulin receptors
  - Type 2: Impaired insulin secretion (compared to the elevated level of glucose in the blood)

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Introduction

- Negative effects of type 2 diabetes -

- Elevated levels of plasma insulin, glucose and fatty acids
- The disease will cause the number and function of β-cells to decline

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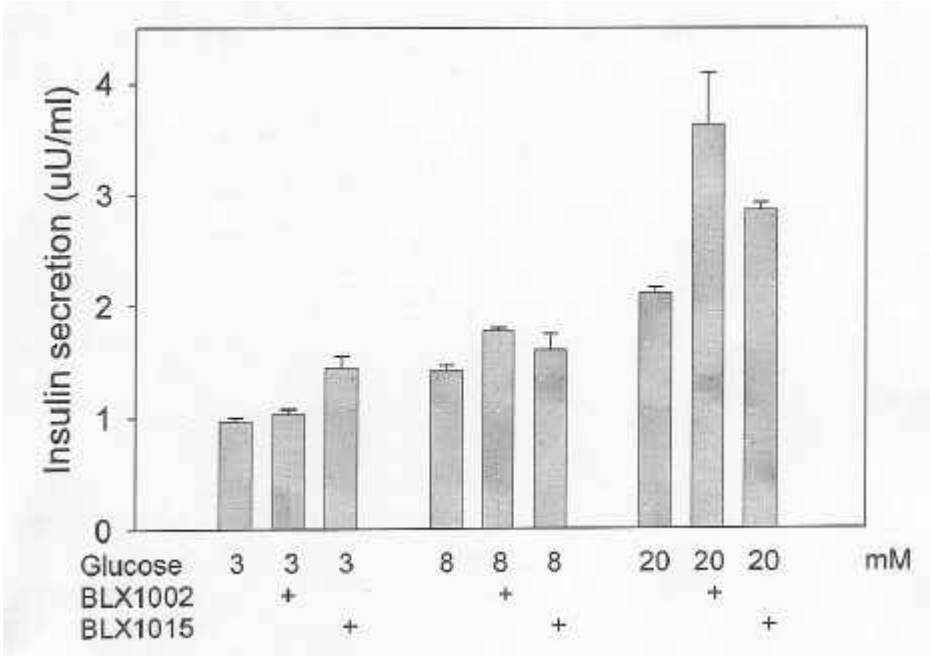
Introduction: BLX-1002

- Candidate drug for treatment of diabetes
- Stimulates insulin release in vivo at high levels of blood glucose, but not at low levels
- Protects the pancreatic islets against the diabetic toxins (in vivo)
- Lowers the levels of glucose, free fatty acids and cholesterol in animal models
- Has a unique anti-weight gain property

Introduction: BLX-1002



This is a preliminary result from the Diabetes group at KISÖS, Stockholm. The bar chart represents the insulin secretion from pancreatic b-cells from ob/ob mice (060110-12). The cells were incubated in the presence of BLX-1002 (10mM) or BLX-1015 (an analogue structurally related to BLX-1002, 10mM) or vehicle (DMSO) at three different concentrations of glucose (3mM, 8mM and 20mM) for 20 minutes, at 37°C. BLX-1002 converts to BLX-1015 after oral dosing.

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Introduction: AMPK

- AMP-activated protein kinase
- Activated by phosphorylation at a low AMP/ATP ratio (i.e. low glucose levels)
  - Turns off energy consuming processes
  - Stimulates energy producing processes
  - Metabolic effects mediated through regulation on gene expression
  - Inhibits insulin secretion

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Introduction: PI3K

- Phosphoinositide-3-kinase
- Activated by phosphorylation at low glucose levels (3 phosphorylation sites)
  - phosphorylates phosphatidyl inositol lipids at the D-3 position on the inositol ring
  - Possible local responses are polymerisation of actin, assembly of signalling complexes and priming of protein kinase cascades.
  - Defects in one of these pathways may cause type 2 diabetes
  - Inhibits insulin secretion
- Inhibition of PI3K blocked BLX-1002 induced insulin secretion

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Matherials and methods

- Stimulation of MIN6 cells and ob/ob mouse beta-islet cells with BLX-1002

- Measuring of the amount of Pi-AMPK and Pi-PI3K with Western blotting

Matherials and methods

- cells-

- MIN6 is a mouse insulin-secreting cell line
- MIN6 cells are tumour cells

- Ob/ob mice are obese
- Ob/ob islets are big and 90% of the islet cells are beta-cells (insulin producing cells)

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Matherials and methods

- MIN6 cell stimulation -

Sample	1	2	3	4	5	6	7	8
Glucose	3mM	20 mM	20 mM	20 mM	20 mM	20 mM	20 mM	20 mM
BLX-1002				10 µM	10 µM	10 µM	10 µM	
Metformin								1 mM
Time (min)	5	5	60	5	15	30	60	60

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Matherials and methods

- Ob/ob mouse islet cells -

Sample	1	2	3	4	5	6	7	8
Glucose	3mM	3 mM	20 mM	20 mM	20 mM	20 mM	20 mM	20 mM
BLX-1002					10 µM	10 µM	10 µM	
Metformin								1 mM
Time (min)	5	60	5	60	5	15	60	60

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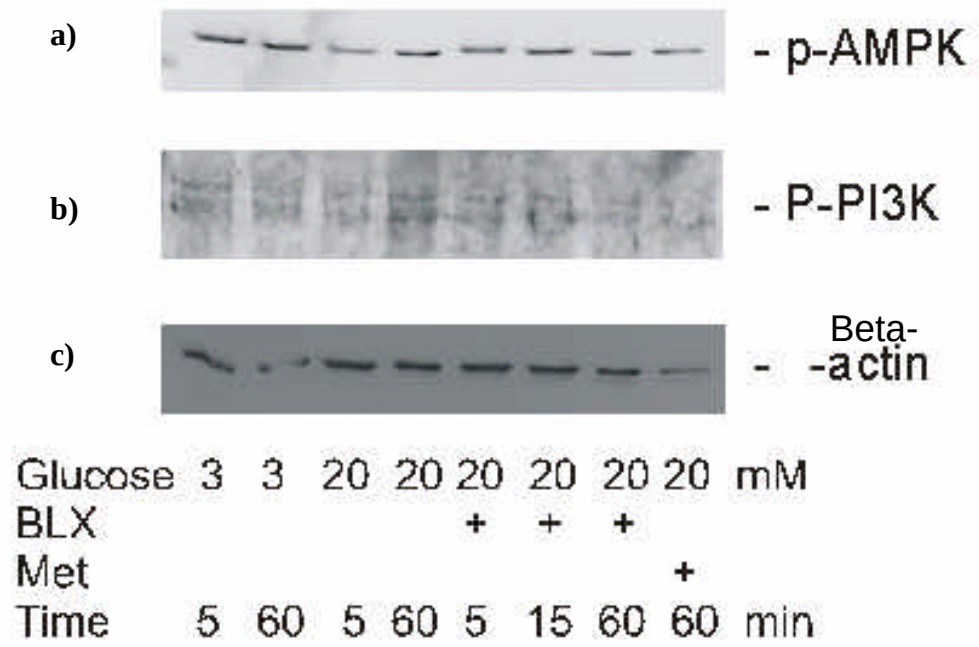
Matherials and methods

- Anti-bodies for western blot:
  - Anti-Pi-AMPK (Threonine phosphorylation)
  - Anti-Pi-PI3K (Tyrosine phosphorylation)
  - Anti-beta-actin
    - Measures total amount of protein in the samples

- The total amount of protein was also measured with a protein assay kit

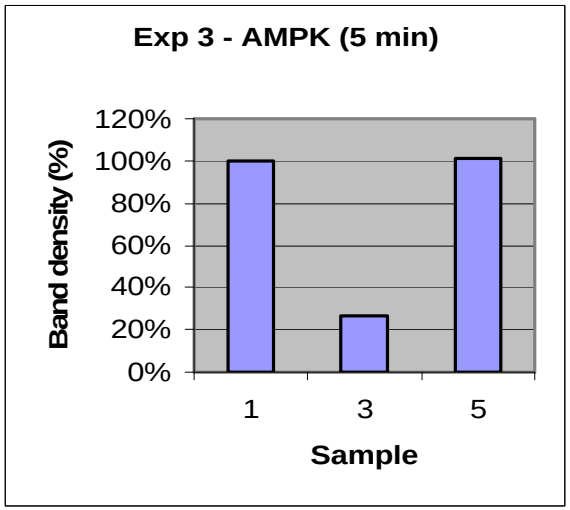
Results: Ob/ob mouse islet cells
- Exposed membrane -



*These are pictures of an exposed membrane from the experiment on ob/ob mouse. Picture **a)** shows the exposure for P_i-AMPK, picture **b)** represents P_i-PI3K and picture **c)** represents b-actin (overall protein amount).*

Results:Ob/ob mouse islet cells
- AMPK (5 min) -

a)



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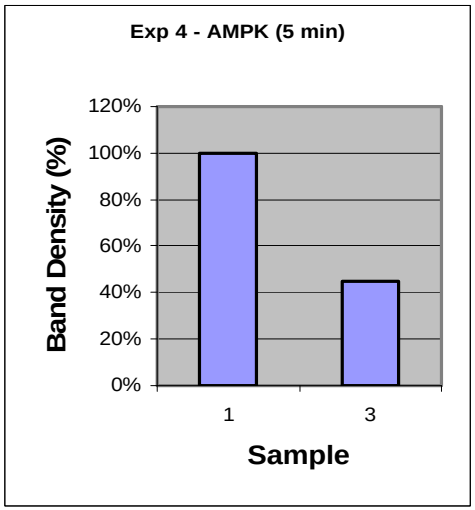
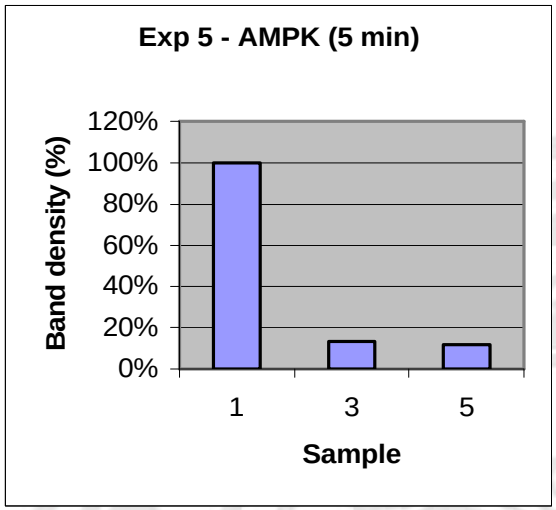


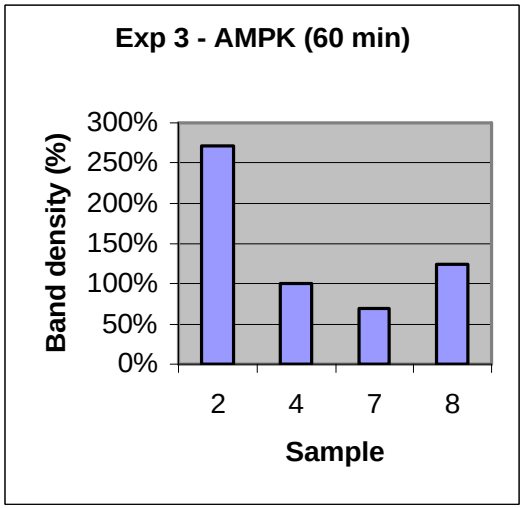
c)



The bar charts above represent all the samples with an incubation time of 5 minutes from the Western blot experiment on AMPK. Sample 1 is 3 mM Glucose, sample 3 is 20 mM glucose and sample 5 is 20 mM glucose with 10mM BLX-1002. The data are normalised with the results from the protein assay.

Results: Ob/ob mouse islet cells - AMPK (60 min) -

a)



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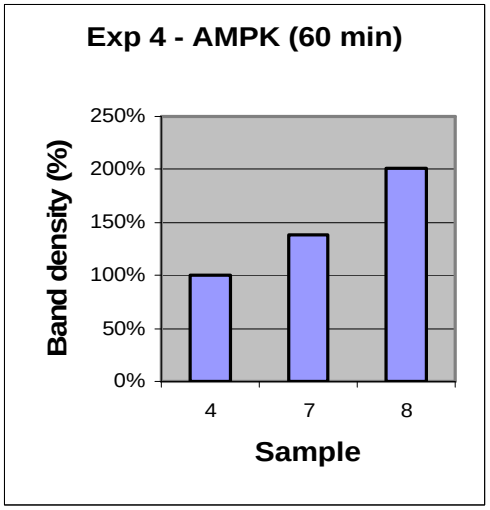

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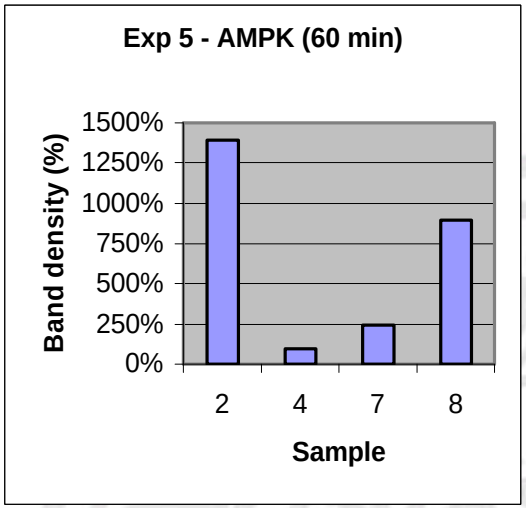
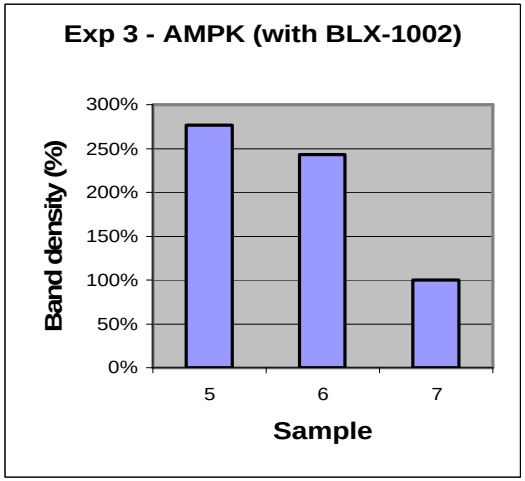


The bar charts above represent all the samples with an incubation time of 60 minutes from the Western blot experiment on AMPK. Sample 2 is 3mM Glucose, sample 4 is 20 mM glucos, sample 7 is 20 mM glucose with 10mM BLX-1002 and sample 8 is 20 mM glucose with 1 mM metformin. The data are normalised with the results from the protein assay.

Results: Ob/ob mouse islet cells - AMPK (with BLX-1002) -

a)



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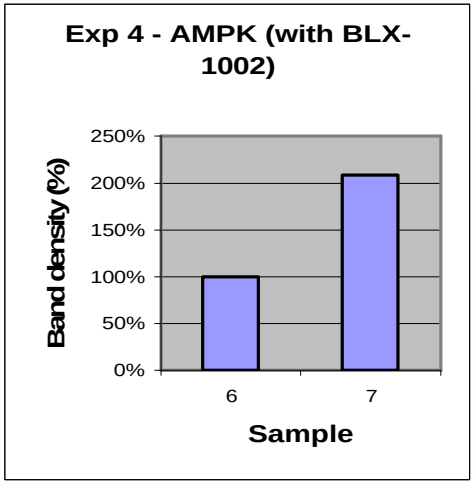

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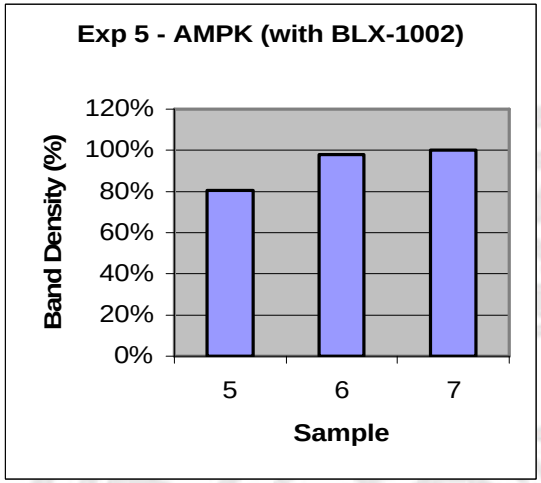

The bar charts above represent all the samples from the Western blot experiment on AMPK that was incubated with 10 mM BLX-1002 (at 20 mM glucose). Sample 5 was incubated for 5 minutes, sample 6 was incubated for 15 minutes and sample 7 was incubated for 60 minutes. The data are normalised with the results from the protein assay.

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Discussion: AMPK - Controls -

- High activity at low glucose
- Low activity at high glucose
- High activity with metformin at high glucose

- Conclusion: The cells reacted to the stimulation and the results are trustworthy

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Discussion:AMPK

- Result: Sustained activation of AMPK by BLX-1002 (at least 60 minutes).

- AMPK inhibits glucose-induced insulin release
- BLX-1002 stimulates insulin release

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Discussion: AMPK

- AMPK regulates cellular metabolism through Gene Expression
- BLX-1002 has other effects:
  - lowers the levels of glucose, free fatty acids and cholesterol
  - a unique anti-weight gain property.

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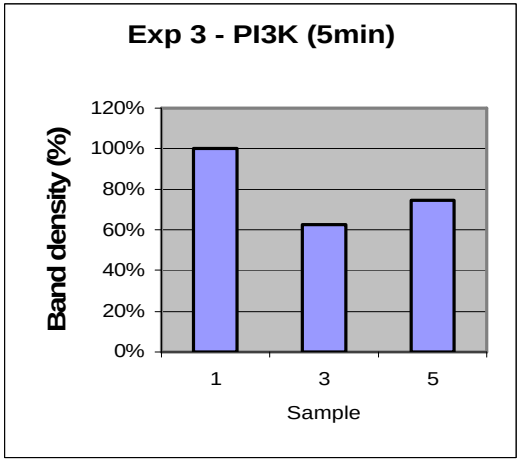
Discussion: AMPK

- AMPK may not be involved in the stimulation of insulin release caused by BLX-1002
- AMPK may instead be involved in the metabolic actions of the drug.

Results: Ob/ob mouse islet cells - PI3K (5 min) -

a)



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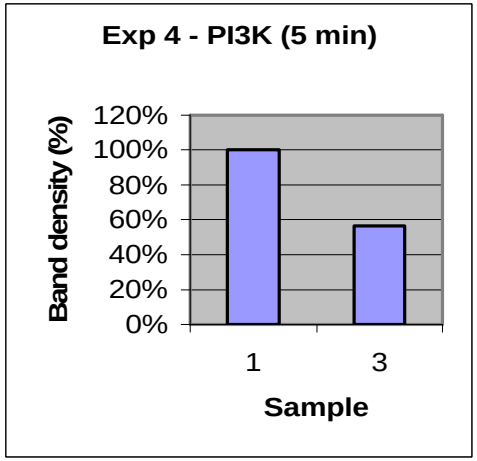

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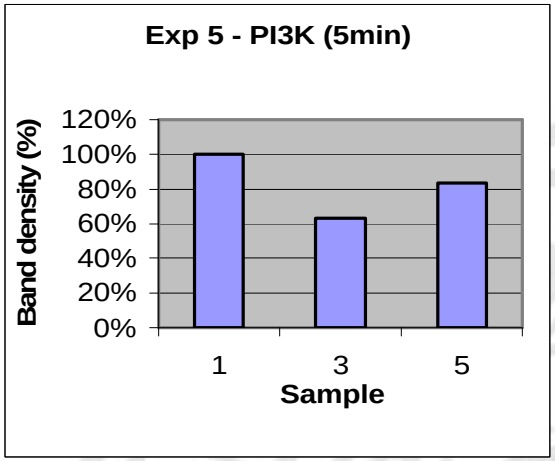
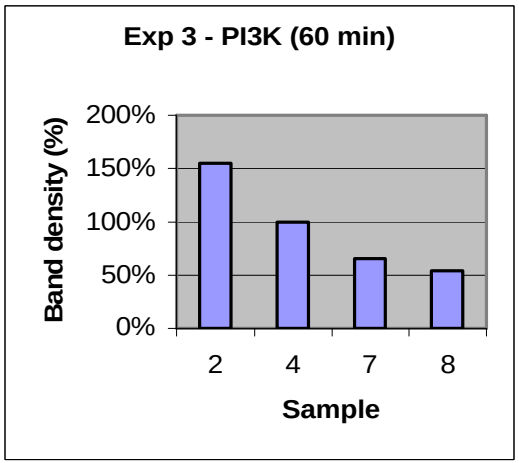


The bar charts above represent all the samples with an incubation time of 5 minutes from the Western blot experiment on PI3K. Sample 1 is 3mM Glucose, sample 3 is 20 mM glucose and sample 5 is 20 mM glucose with 10mM BLX-1002. The data are normalised with the results from the protein assay.

Results: Ob/ob mouse islet cells - PI3K (60 min) -

a)



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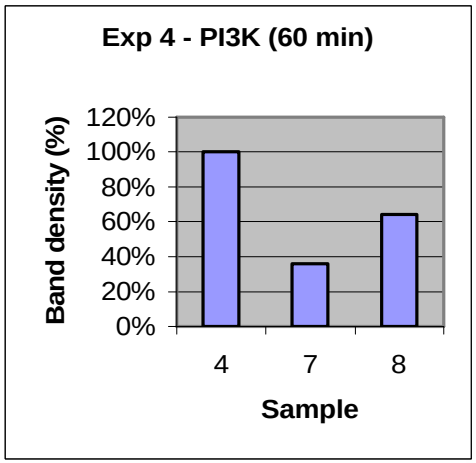

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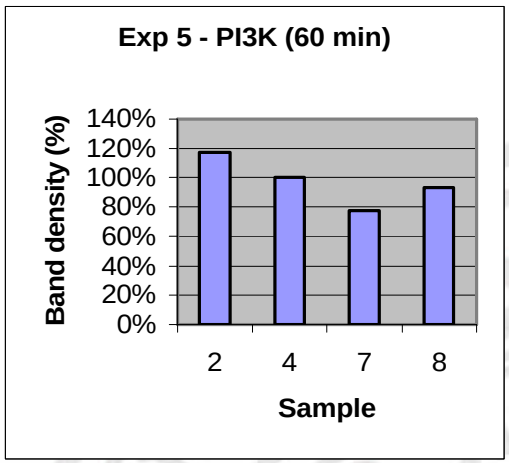
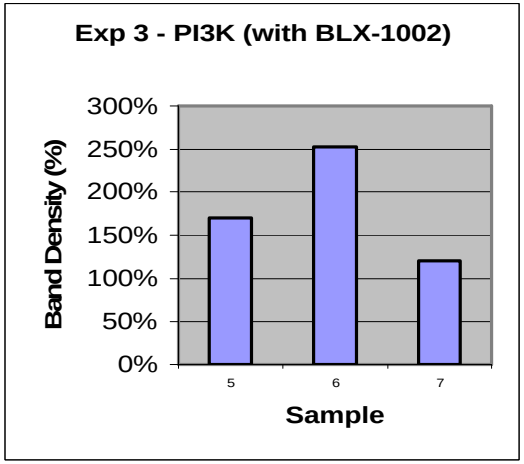


The bar charts above represent all the samples with an incubation time of 60 minutes from the Western blot experiment on PI3K. Sample 2 is 3mM Glucose, sample 4 is 20 mM glucose, sample 7 is 20 mM glucose with 10mM BLX-1002 and sample 8 is 20 mM glucose with 1 mM metformin. The data are normalised with the results from the protein assay.

Results: Ob/ob mouse islet cells
- PI3K (with BLX-1002) -

a)



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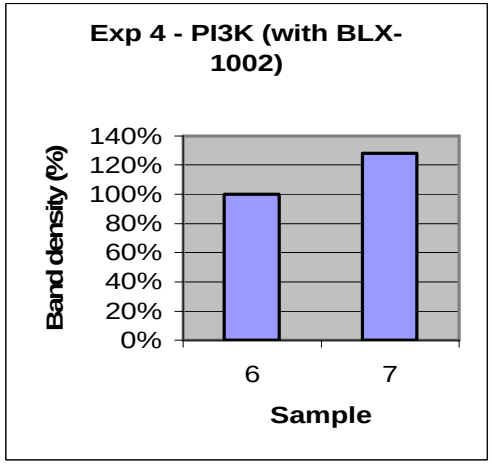

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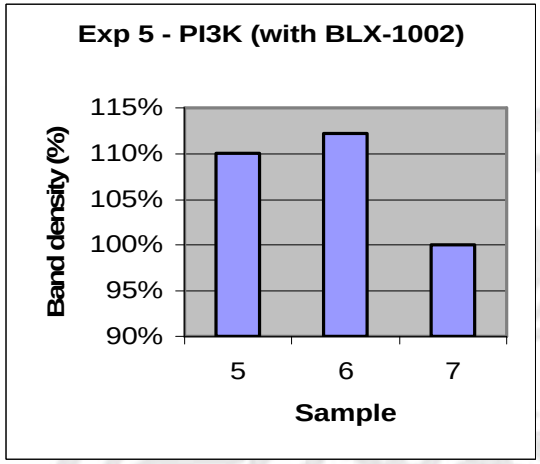

The bar charts above represent all the samples from the Western blot experiment on PI3K that was incubated with 10 mM BLX-1002 (at 20 mM glucose). Sample 5 was incubated for 5 minutes, sample 6 was incubated for 15 minutes and sample 7 was incubated for 60 minutes. The data are normalised with the results from the protein assay.

Discussion: PI3K
- controls -

- High activity at low glucose
- Low activity at high glucose

- The cells seem to have responded to the stimulation

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Discussion: PI3K

- Temporary activation of PI3K by BLX-1002
- Inhibition of PI3K by BLX-1002 after 60 minutes
- PI3K inhibits glucose-induced insulin release
- BLX-1002 stimulated insulin release
- Insulin release stimulated by BLX-1002 was blocked when PI3K was inhibited

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Discussion: PI3K

- PI3K seems to be required for insulin release stimulated by BLX-1002

- Both negative and positive evidence against a requirement for PI3K in insulin secretion

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Discussion: PI3K

- Further research on the role of PI3K in insulin secretion needs to be performed

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Conclusion

- BLX-1002 tends to activate both AMPK and PI3K
- Sustained activation of AMPK
- Temporary activation of PI3K followed by an inhibition of the enzyme
- Activation of AMPK induced by BLX-1002 may be involved in the metabolic effects of the drug
- PI3K seems to be necessary for stimulated insulin release caused by BLX-1002

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The end

Thank you for listening.

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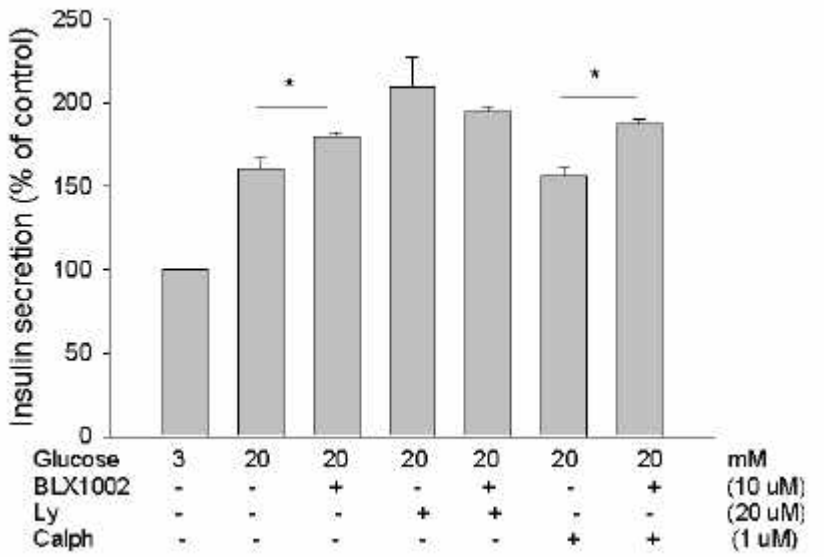
BLX-1002

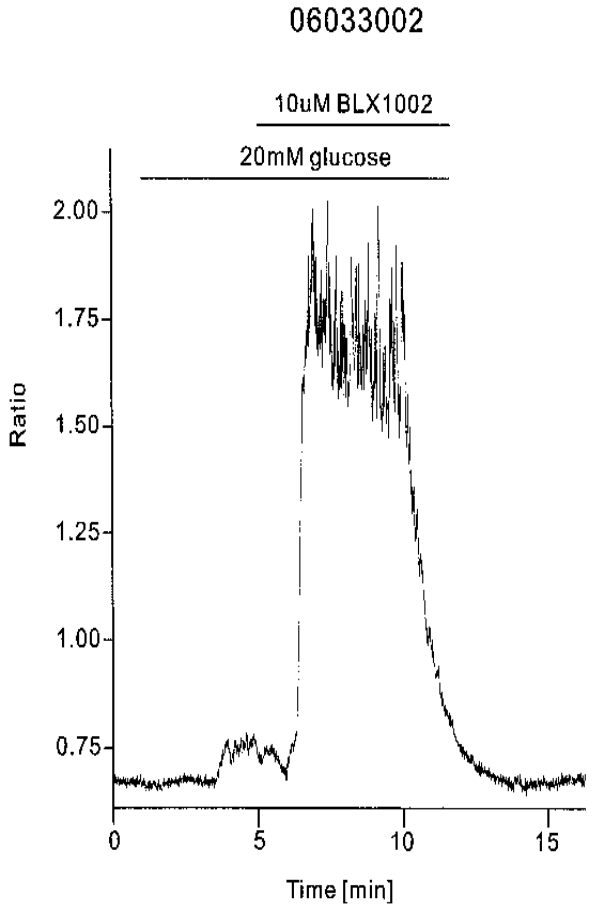
- An amino acid conjugated, water-soluble molecule
- Molecular mass 500 Dalton
- No structural resemblance to any existing anti-diabetic compounds
- Has completed safety toxicity pre-clinical studies in the US, in both lower order animals like rodents and higher order animals like dogs
- Has completed safety studies in healthy and diabetic patients

Introduction: BLX-1002



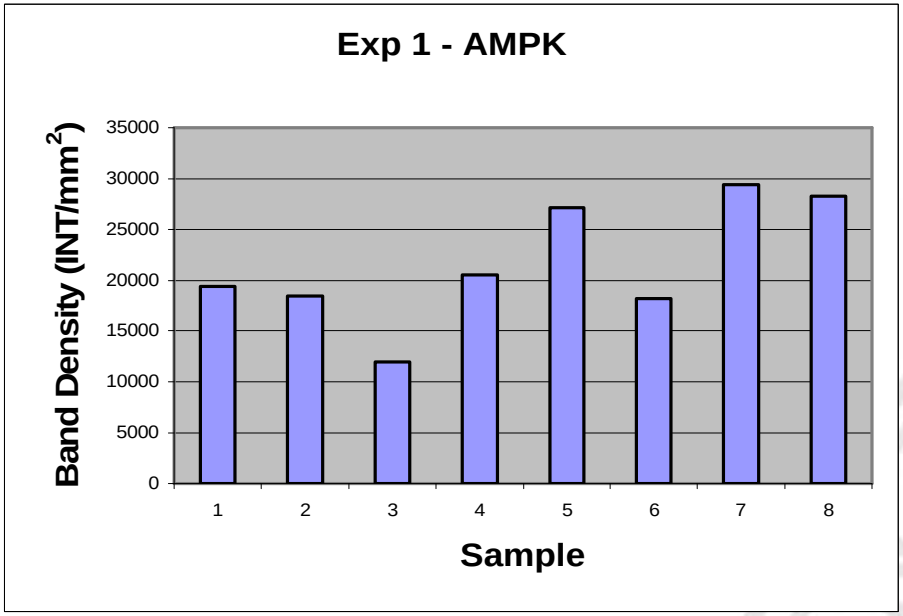
The bar chart represents a preliminary result from the Diabetes group at KISÖS in Stockholm, where insulin secretion from ob/ob mouse b-cells have been measured. Cells were pre-incubated in the presence of LY29400 (a specific PI3K inhibitor, 20 mM), Calphostin C (a specific PKC inhibitor, 1 mM) or vehicle for 15 min, followed by addition of BLX-1002. Incubation was initiated by addition of glucose and lasted for 20 min, at 37 °C. A triplicate experiment is shown (P ? 0.05 by ANOVA).*

Introduction: BLX-1002



This is a preliminary result from the Diabetes group at KISÖS, Stockholm. The graph indicates the level of intracellular Ca^{2+}. The measurement starts out at a low glucose level. Glucose is then added to increase the concentration to 20 mM. BLX-1002 is then added at a concentration of 10mM.

Results: MIN6 cells



This is the bar chart from the first repetition of the MIN6 experiment (AMPK). Sample 1 represents low glucose and 5 minutes incubation; sample 2 is high glucose and 5 minutes incubation; sample 3 is high glucose and 60 minutes incubation, sample 4 is high glucose with BLX present and 5 minutes incubation; sample 5 is high glucose with BLX-1002 present and 15 minutes incubation, sample 6 is high glucose with BLX-1002 present and 30 minutes incubation, sample 7 is high glucose with BLX-1002 present and 60 minutes incubation and sample 8 is high glucose with metformin present and 60 minutes incubation. The data is normalised with the results from the protein assay.

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Discussion: PI3K

- PI3K has been shown to suppress glucose stimulated insulin secretion by affecting the levels of intracellular Ca^{2+} .
- PI3K inhibitors have been shown to increase insulin secretion in 832/13 rat insulinoma cells

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Discussion: PI3K

- Indications that PI3K inhibitors amplify the release of insulin in lean mice, but not in obese mice
- In mice lacking the p110gamma isoform of PI3K, glucose stimulated insulin secretion was impaired
- L-783,281 evokes intracellular [Ca^{2+}] increases and Ca^{2+}-exocytosis in β-cells via an IRS-1/**PI3K**-dependent pathway
- Leptin constrains phospholipase C-protein kinase C-induced insulin secretion through a PI3K-dependent pathway