

COMPOUND B

Compound B is a potential anticancer agent which had previously been studied using traditional biochemical approaches. These had suggested that it might act via one of the peroxisome proliferator activated family of nuclear receptors (PPARs). CXR Biosciences undertook a series of studies to elucidate the mechanism of action of Compound B, including target validation, characterisation of novel signalling pathways and the identification of pharmacodynamic markers for use in clinical trials. As a result of these studies, Compound B is now poised to enter Phase I clinical trials.

Interaction with recombinant PPARs.

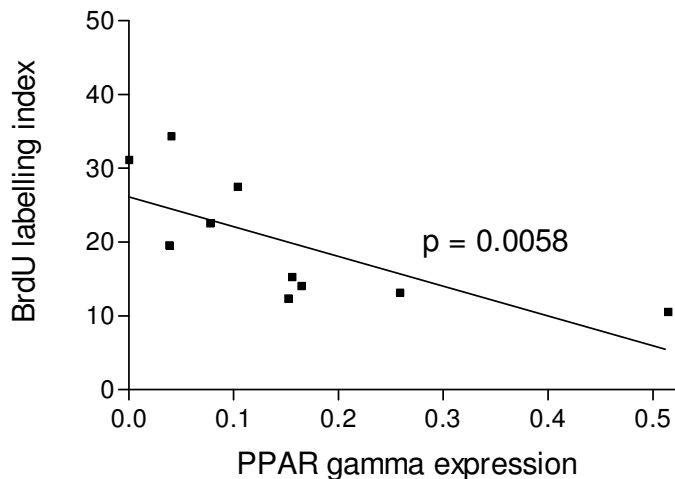
It was well known from classical biochemical and toxicological approaches that Compound B could act as a PPAR agonist in rodents. However, a more incisive approach was needed to demonstrate its ability to bind to human receptors which could mediate its anticancer effects. We took advantage of recombinant technology to create chimeric PPAR constructs which could be used to characterise the effects of Compound B. We adopted two strategies:

- In the GAL4 binding assay, a chimeric construct containing the ligand binding domain of the PPAR of interest linked to the DNA binding domain of the GAL4 transcription factor was used. This assay was used to demonstrate the ability of compounds to bind to the PPAR ligand binding site and cause a conformational change leading to receptor activation.
- In the transactivation assay, the full length PPAR was expressed in Cos1 cells and used to demonstrate the ability of the PPAR to activate transcription of target genes, as detected using a luciferase reporter gene containing a peroxisome proliferator-responsive element (PPRE).

The GAL4 and transactivation assays indicated that Compound B can bind to both PPAR α and PPAR γ leading to a conformational change consistent with receptor activation.

Role of PPARs in the response to Compound B

In order to determine which PPAR mediated the effects of Compound B, the Spearman Rank Correlation method was used to search for correlations between PPAR expression levels (measured using TaqMan Real Time PCR) and effects on cytotoxicity, BrdU labelling index and apoptotic index *in vitro*. A correlation was detected between PPAR γ expression and BrdU labelling index, which decreased with increasing PPAR γ expression, suggesting that PPAR γ is involved in the regulation of cell turnover by Compound B in human tumour-derived cell lines *in vitro*.



Growth inhibition by PPAR γ ligands has been observed in cell lines derived from a number of tumour types. In addition to their direct effects on the cell cycle, PPAR γ ligands have indirect effects on the rate of tumour

growth and are potent inhibitors of angiogenesis and cell-cell communication *in vivo* and *in vitro*. Terminal differentiation is also thought to play a key role in their growth inhibitory effects. Furthermore, PPAR γ agonists induce apoptosis in tumour cells. The apoptotic processes involved appear to involve both caspase-dependent and independent mechanisms.

PPAR γ agonists have already been shown to have therapeutic benefit in cancer. Clinical trials have indicated a reduction in PSA levels by troglitazone in ~ 20% of prostate cancer patients, and studies in breast cancer have suggested that PPAR γ may have a role in chemoprevention of cancer as well as therapy.

Transcriptional profiling in xenografts

Recombinant receptor studies and work with cancer cell lines showed that Compound B could regulate gene expression via PPAR γ , but for Compound B to have a future as an anticancer agent it was critical to demonstrate effects in human tumour cells *in vivo*. We used xenograft methodology to grow PC3 human prostatic tumour cells in nude mice and then carried out transcriptional profiling to demonstrate that the compound had direct anticancer effects on the tumour cells. These findings helped us to understand the anticancer effects of Compound B as well as leading to the development of biomarkers useful in the pre-clinical and clinical development of Compound B.

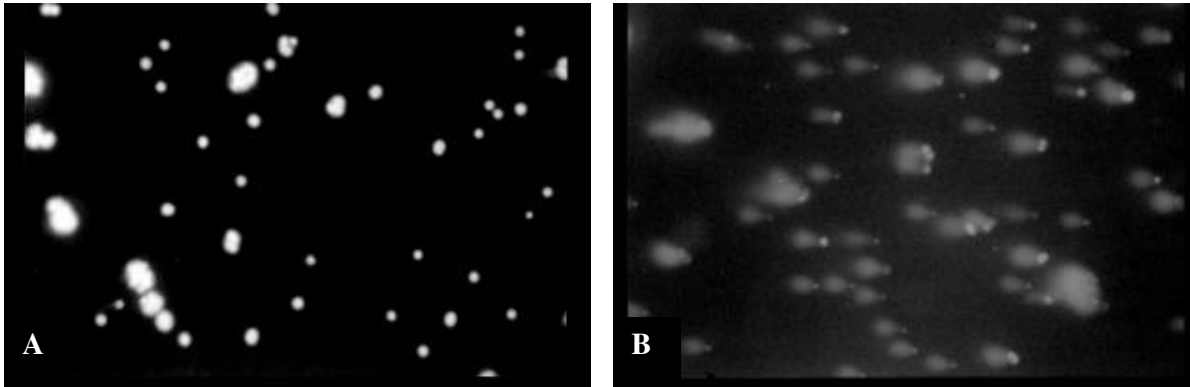
Anti-tumour drugs act through inhibition of tumour cell proliferation and/or increasing the rate of tumour cell death or differentiation. We focussed our attention on novel pathways which could provide plausible insights into the mechanism of anti-tumour activity of Compound B. Two novel pathways were identified; one was predicted to enhance cell death processes while the other was associated with the modulation of differentiation pathways. The transcriptional profile of Compound B indicated perturbations in the expression of transcription factors and numerous alterations in expression of proteins involved in pathways leading to a terminally differentiated phenotype. Cells with these characteristics would have a lower proliferative capacity than untreated prostate tumour cells, thus contributing to the anti-cancer effects of Compound B.

Potential carcinogenic liability

These studies were encouraging in terms of the development of Compound B as an anticancer drug; however, a concern was raised because some PPAR agonists can induce tumours in experimental animals. The carcinogenicity of Compound B had previously been investigated using traditional methods such as two-year feeding studies in rats, in which increased incidences of liver, Leydig cell, and pancreatic acinar cell tumours were observed. Compound B is negative in a range of *in vitro* assays for DNA damage, including the Ames test, and in the mouse micronucleus assay. Now, if Compound B is truly non-genotoxic, the mechanisms of tumour induction in rats may not be of relevance to man. We therefore set out to verify that Compound B is, indeed, non genotoxic and to elucidate its mechanism of action.

The Comet assay

A particular cause for concern was the ability of Compound B to induce preneoplastic foci in rat pancreatic acini, with the possibility that these could develop into frank adenocarcinomas. In order to find out whether Compound B causes these foci via a genotoxic action, we used the Comet assay (single cell gel electrophoresis) to examine isolated pancreatic acinar cells from rats treated *in vivo* with Compound B. This assay allows the detection of both single- and double-stranded breaks in cellular DNA, seen as “comet tails” streaming away from the nucleus of the cell. No comets were seen in cells from rats treated with Compound B, whereas the positive control clearly showed the characteristic pattern indicating breaks within DNA.



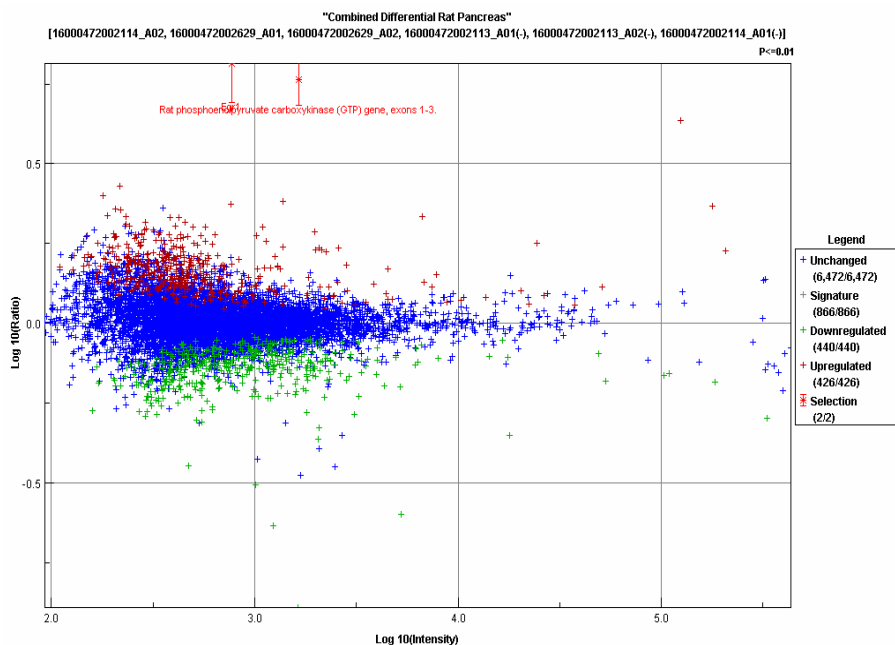
DAPI-stained comet nuclei following electrophoresis of pancreatic acinar cells under alkaline conditions showing 300 ppm Compound B (panel A) vs azaserine (panel B).

This study showed that Compound B was non-genotoxic to rat pancreatic acinar cells. The mechanism by which pre-neoplastic foci are induced in the pancreas by Compound B does not occur as a result of damage to cellular DNA but by an alternative route that requires further investigation.

Transcriptional profiling

The Comet assay evidence substantiated the hypothesis that Compound B is not directly genotoxic in rat pancreas. In order to further develop our ideas about potential non-genotoxic mechanisms of pancreatic carcinogenesis, the powerful technique of transcriptional profiling was used. Differential gene expression associated with Compound B treatment was assessed by performing competitive hybridisations of labeled cDNAs from test and control tissue samples on a printed cDNA microarray. This allowed us to look at 14,000 rat genes simultaneously and showed that 267 pancreas genes and 294 liver genes were up-regulated and 310 pancreas and 323 liver genes were down-regulated.

Graphical representation of pancreatic microarray data. Red and green dots represent those genes selected as significantly up- or down-regulated respectively, according to a 99% confidence ($P < 0.01$) level. Two genes which show the the highest fold changes (up-regulation) are highlighted.



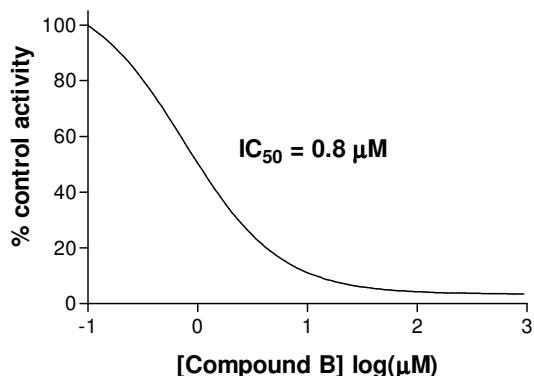
Examining the transcriptional effects of Compound B in the liver generated a profile of changes consistent with increases in hepatic β -oxidation and fat metabolism, suggesting that, in the rat liver, Compound B is acting as a PPAR α agonist and peroxisome proliferator. By contrast gene expression changes observed in the pancreas were quite different, suggesting effects on gluconeogenesis and glutamine metabolism. The profile of regulated genes associated with carcinogenesis was indicative of metabolic acidosis, oxidative stress and endogenous processes of DNA mutation against a background of increased mitogenesis.

It is likely that cancer-related gene expression changes observed in this study are early events in the carcinogenic process. Genes involved in MAP kinase and Notch signaling, together with target genes for these signalling pathways, were up-regulated. Gene expression changes which could lead to metabolic acidosis and perturbations in glutamine metabolism and apoptosis were also observed. A combination of increases in endogenous processes of mutation with increased cell proliferation could form the basis of a non-genotoxic mechanism of Compound B-induced carcinogenesis in the pancreas.

Overall, then, Compound B seems to be acting as a “classical” PPAR α agonist in the rat liver but more like a PPAR γ agonist in the pancreas. In addition, as might be expected from a compound able to activate these receptors, which are involved in a wide range of metabolic processes relating to energy and lipid homeostasis, Compound B has profound effects on cellular metabolism in both tissues. These data are reassuring because there is good evidence that PPAR α -mediated liver carcinogenesis is a rodent-specific phenomenon which is unlikely to occur in man, and PPAR γ is, if anything, associated with anticarcinogenic effects. This single experiment was able to provide safety information which would have taken years to collect by traditional toxicological methods.

Preparation for clinical trials

Having identified possible mechanisms of action for Compound B, and reassured the sponsor that no carcinogenic liability was likely to be incurred by the use of this compound in therapy, preparations began to take the compound into clinical trials. Before administering Compound B to humans, its bioavailability,



metabolism and ability to inhibit key drug metabolising enzymes were evaluated. Compound B turned out to be highly bioavailable and was metabolically stable; however, it did turn out to be a potent inhibitor of one of the cytochrome P450 (CYP) family of Phase I drug metabolising enzymes. In an *in vitro* cassette assay using five different recombinant CYPs we observed significant inhibition of CYP2C9 with an IC₅₀ value of 0.8 μ M. No significant inhibition of the other four CYP isoforms was observed. This observation enabled us to give critical advice to the sponsor that they should avoid coadministration of Compound B with warfarin, NSAIs, phenytoin and tolbutamide.

Conclusion

The application of modern techniques from our molecular biology portfolio has enabled us to acquire, within a matter of months, powerful insights into the mechanism of action of Compound B as well as its therapeutic potential. We have ruled out carcinogenic and metabolic liabilities, defined exclusion criteria to ensure the safety of trial volunteers and identified pharmacodynamic markers. Armed with these data, Compound B is now ready to proceed to Phase I clinical trials. With traditional approaches many of the possible targets might never have been identified and, even if they were, the data would have taken years to acquire.