# Chapter 4 – Investigation of the Upstream Mechanism to Activation of NF-κB by NSAIDs

## 4.1 Introduction

It is well established that cytokines, such as TNF, activate the NF-κB pathway through phosphorylation of IκBα on serine residues at positions 32 and 36. This results in degradation of IκBα by ubiquitin-mediated proteolysis (see Chapter 1). However, an alternative pathway of activation of NF-κB has been identified. Activation of NF-κB by agents such as pervanadate (Imbert *et al.*, 1996; Mukhopadhyay *et al.*, 2000), hypoxia/reoxygenation (Imbert *et al.*, 1996; Fan *et al.*, 2003) and/or oxidative stress (Schoonbroodt *et al.*, 2000) involves phosphorylation of IκBα on tyrosine 42. Unlike the classical pathway, involving serine phosphorylation of IκBα, activation of NF-κB by tyrosine phosphorylation of IκBα has been shown to occur in the absence of IκBα degradation (Imbert *et al.*, 1996; Singh *et al.*, 1996; Bui *et al.*, 2001). Under those circumstances, the dissociation of IκBα from NF-κB occurs via an unknown mechanism.

The tyrosine kinase responsible for phosphorylation of IκB at Tyr 42 has not been defined, but several candidates have been proposed and these include: $p56^{lck}$ (Imbert *et al.*, 1996; Livolsi *et al.*, 2001; Mahabeleshwar and Kundu, 2003); ZAP-70 (Livolsi *et al.*, 2001); c-Abl (Kawai *et al.*, 2002); Syk (Takada *et al.*, 2003) and c-Src (Abu-Amer *et al.*, 1998; Fan *et al.*, 2003; Kang *et al.*, 2006).

c-Src is an attractive candidate in light of considerable emerging evidence of a role in colorectal cancer progression [reviewed in (Irby and Yeatman, 2000; Frame, 2002; Warmuth *et al.*, 2003)]. It is a member of a closely related family of non-receptor membrane-associated protein tyrosine kinases. The Src family kinases (SFKs), which include Src, Fyn, Yes, Blk, Yrk, Fgr, Hck, Lck and Lyn, participate in regulation of diverse functions such as proliferation, cell cycle progression, migration, adhesion, and differentiation [reviewed in (Thomas and Brugge, 1997; Schlessinger, 2000)]. c-Src is a particularly good candidate for activation of the NF-κB pathway by NSAIDs as two studies (Abu-Amer *et al.*, 1998; Fan *et al.*, 2003) have demonstrated that c-Src directly phosphorylates IκBα and c-Src has been shown to directly interact with IκBα (Abu-Amer *et al.*, 1998).

In view of the observation presented in Chapter 3 that non-aspirin NSAIDs activate NF-κB without degradation of IκBα, the aim of this chapter was to specifically investigate whether tyrosine phosphorylation of IκBα is implicated the upstream mechanism to activation of the NF-κB pathway by non-aspirin NSAIDs.

## 4.2 Results

### 4.2.1 Detection of Tyrosine Phosphorylated IκBα

The first step was to determine whether IκBα was in fact phosphorylated on tyrosine 42 in response to NSAID exposure. To that end, SW480 cells were treated for 16 hrs with aspirin, sulindac, sulindac sulfone and indomethacin prior to

harvesting. Immunoprecipitation of IκBα, followed by Western blot analysis with an anti-phospho-tyrosine antibody, was carried out using standard methods (see Chapter 2). Although tyrosine phosphorylated proteins were detected in some input tracks (see black arrows) and Western blotting for IκBα (non tyrosine phosphorylated) confirmed that IκBα was specifically immunoprecipitated from the extract, there was no evidence for tyrosine phosphorylation of IκBα in response to any of the NSAIDs on probing with anti-phospho-tyrosine antibody (Figure 4.1).

It has previously been shown in Jurkat cells (T-cell leukaemia line) that tyrosine 42 phosphorylation of IκBα occurs 30-60 mins following exposure to pervanadate (Imbert *et al.*, 1996). Hence, one possibility was that 16 hrs of treatment with the NSAIDs was too long a time point for detection of tyrosine phosphorylated IκBα. Therefore, tyrosine phosphorylation of IκBα after 3 or 5 hrs treatment with sulindac or 5 hrs with pervanadate, as a positive control, was the next stage of investigation. Although Figure 4.2 demonstrates that tyrosine phosphorylation of IκBα was detectable after pervanadate treatment in one experiment (see green arrow), this result was not reliably replicated. This suggests one of three possibilities: SW480 cells do not respond to pervanadate as reported previously in Jurkat cells; tyrosine phosphorylation may be transient and highly dependent on cell microenvironment, therefore causing difficulties with detection; the method of detection is unreliable. Although no phosphorylated protein was observed after sulindac treatment, tyrosine phosphorylation of IκBα could therefore still have taken place but was just not being detected by the method used. For both pervanadate and sulindac, controls again established that IκBα was specifically immunoprecipitated in an antibody-dependent manner (Figure 4.2).

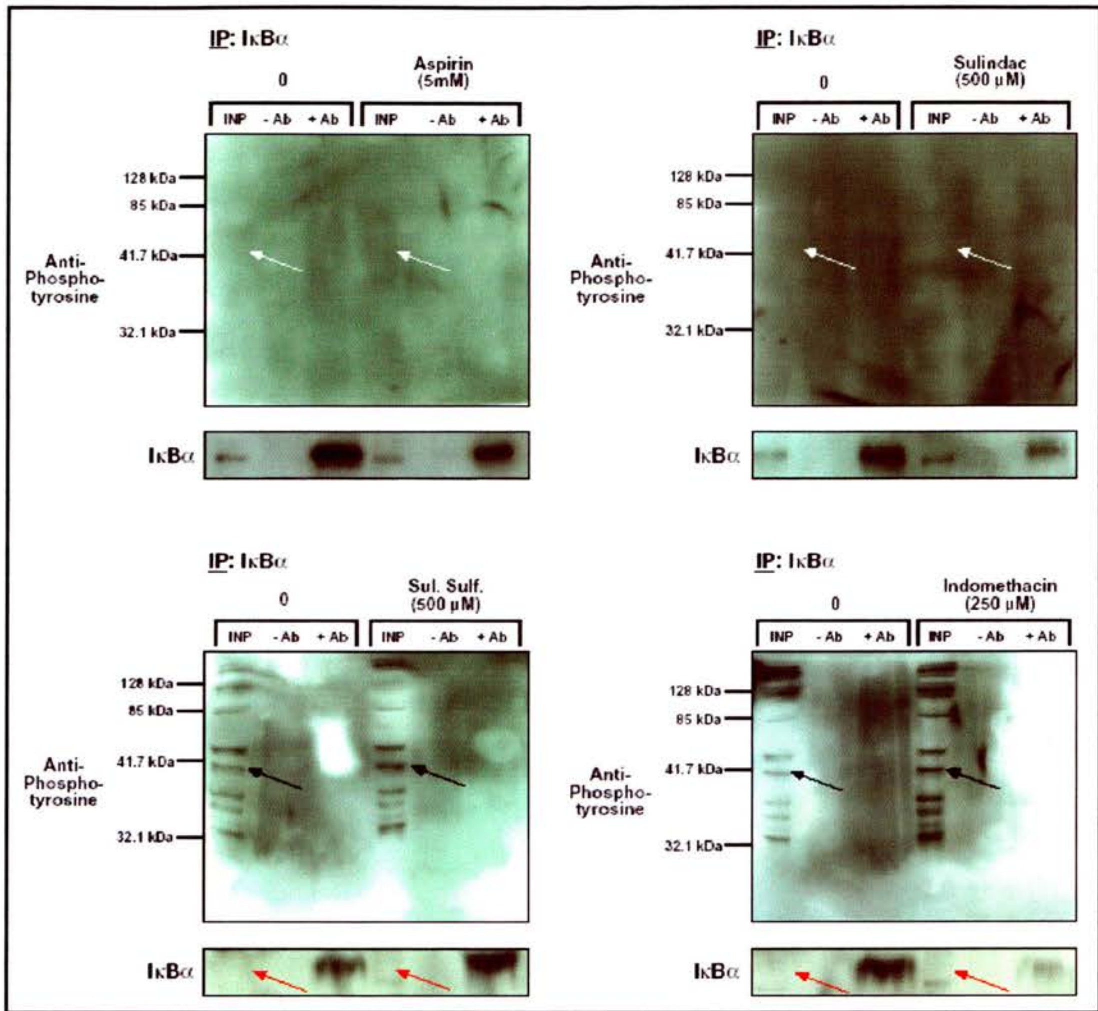



**Figure 4.1 – Assessment of Tyrosine Phosphorylation of IκBα in Response to NSAIDs.**

In three independent experiments, SW480 cells were treated for 16 hrs with Aspirin (0, 5 mM), Sulindac (0, 500 μM), Sulindac Sulfone (Sul. Sulf.) (0, 500 μM) or Indomethacin (0, 250 μM), then whole cell extracts (500 μg) immunoprecipitated (IP) using sheep anti-IκBα polyclonal antibody (+ Ab) or no antibody (- Ab) as a control. Inputs (INP) (30 μg) and immunoprecipitates were resolved by SDS PAGE on 10% polyacrylamide gels and Western blot analysis, using an anti-phospho-tyrosine antibody, carried out. Blots were stripped and re-probed with rabbit polyclonal anti-IκBα antibody. Representative blots are shown. White arrows indicate lack of detection of tyrosine phosphorylated proteins in input tracks; black arrows indicate good detection of tyrosine phosphorylated proteins in input tracks; red arrows indicate lack of detection of IκBα in input tracks.

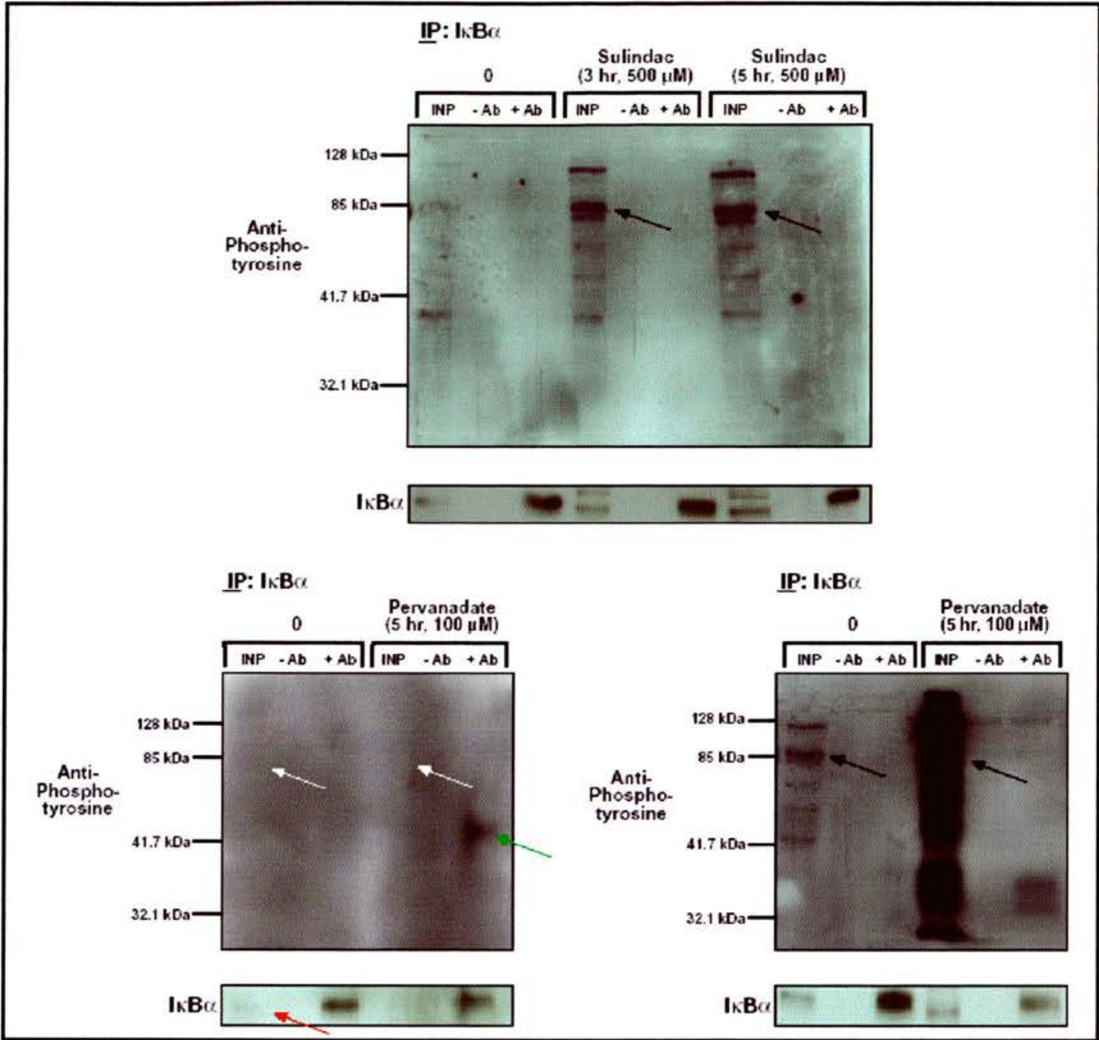
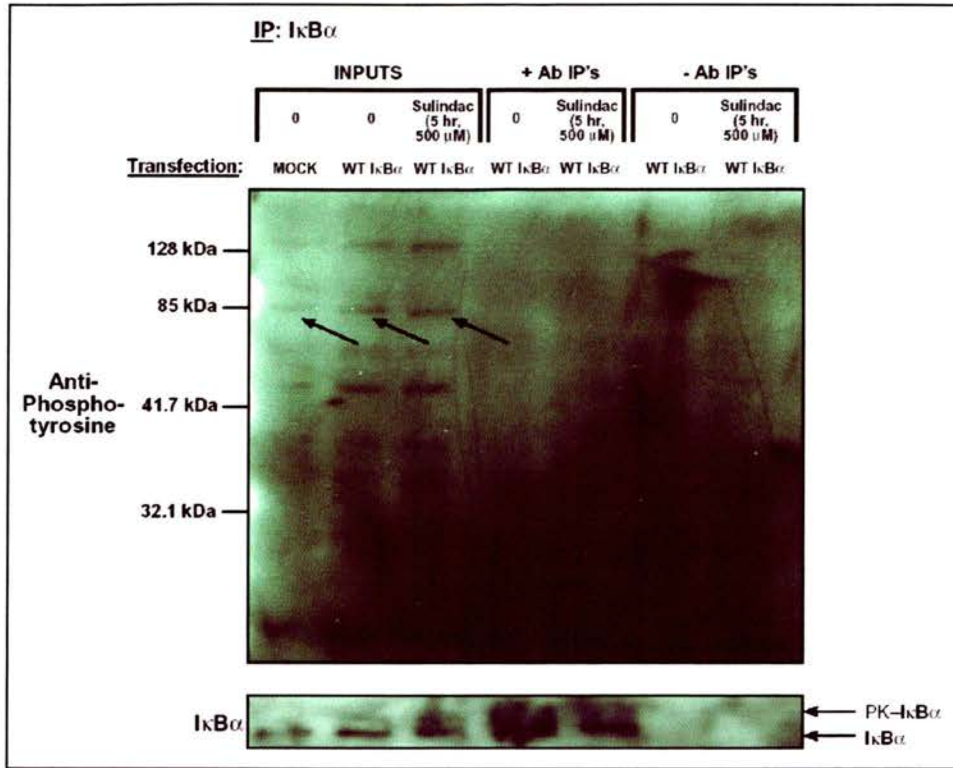



**Figure 4.2 – Assessment of Tyrosine Phosphorylation of IκBα in Response to Short-term Treatment with Sulindac and Pervanadate.** SW480 cells were treated, in three independent experiments, for 3 or 5 hrs with Sulindac (0, 500 μM) or Pervanadate (0,100 μM) then whole cell extracts (500 μg) immunoprecipitated (IP) using sheep polyclonal anti-IκBα antibody (+ Ab) or no antibody (- Ab) as a control. Inputs (INP) (30 μg) and immunoprecipitates were resolved by SDS PAGE on 10% polyacrylamide gels and Western blot analysis, using an anti-phospho-tyrosine antibody, carried out. Blots were stripped and re-probed with rabbit polyclonal anti-IκBα antibody. Representative blots are shown. White arrows indicate lack of detection of tyrosine phosphorylated proteins in input tracks; black arrows indicate good detection of tyrosine phosphorylated proteins in input tracks; red arrows indicate lack of detection of IκBα in input tracks; green arrow indicates tyrosine phosphorylated IκBα.

Previous studies that have examined tyrosine phosphorylation of IκBα required overexpression of WT IκBα (Kawai *et al.*, 2002) and so overexpression using a PK-tagged WT IκBα vector was next used to determine whether the tyrosine phosphorylated form was detectable. SW480 cells were transiently transfected with PK-tagged WT IκBα then treated with sulindac for 5 hrs. Immunoprecipitation of IκBα followed by Western blot analysis with an anti-phospho-tyrosine antibody again revealed no evidence of tyrosine phosphorylation of IκBα after sulindac treatment (Figure 4.3A). Once more, IκBα was specifically immunoprecipitated in an antibody-dependent manner. Western blot analysis using an anti-PK antibody confirmed that PK-tagged WT IκBα had been expressed (Figure 4.3B).

It is noteworthy that tyrosine phosphorylated proteins (see white arrows in aspirin and sulindac blots in Figure 4.1 and pervanadate blot in Figure 4.2) and IκBα (see red arrows in sulindac sulfone and indomethacin blots in Figure 4.1 and pervanadate blot in figure 4.2) were not always detected in the input tracks of blots of these experiments. This was despite multiple experiments and suggests that there were technical problems with antibodies or protein transfer to the membrane. These problems could account for the lack of detection of tyrosine phosphorylated IκBα, but it was still vital to determine definitively whether or not IκBα is tyrosine phosphorylated. Hence, the next strategy was to determine whether tyrosine phosphorylated IκBα in response to sulindac could be detected by mass spectrometry, as this is a much more sensitive detection method for protein modifications. Despite several attempts, neither immunoprecipitated nor recombinant IκBα were detected by mass spectrometry due to technical difficulties.

A

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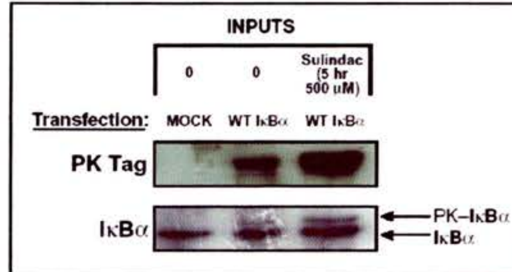



**Figure 4.3 – Assessment of Tyrosine Phosphorylation of Overexpressed WT IκBα in Response to Sulindac Treatment.** SW480 cells were transiently transfected, in three independent experiments, with PCDNA3.1 (MOCK) or WT IκBα prior to treatment with Sulindac (0, 500 μM) for 5 hrs. **(A)** whole cell extracts (500 μg) were immunoprecipitated (IP) using sheep polyclonal anti-IκBα antibody (+ Ab) or no antibody (- Ab) as a control. Inputs (30 μg) and immunoprecipitates were resolved by SDS PAGE on 10% polyacrylamide gels and Western blot analysis, using an anti-phospho-tyrosine antibody, carried out. Blots were stripped and re-probed with rabbit polyclonal anti-IκBα antibody. Representative blots are shown. Black arrows indicate detection of tyrosine phosphorylated proteins in input tracks. **(B)** Anti-PK Western blot and Anti-IκBα Western blot of Inputs (10 μg) from immunoprecipitation experiment in **(A)**. Representative blots are shown.

The above description represents a substantial experimental undertaking, but it was disappointing in terms of its lack of provision of a definitive answer as to whether or not IκBα is phosphorylated on tyrosine 42 in SW480 cells in response to sulindac or the other non-aspirin NSAIDs. Furthermore, the response to pervanadate was also equivocal and so it is unclear as to whether the cells or the agents or the techniques were the problem. I was unable to unambiguously determine the tyrosine 42 phosphorylation status of IκBα in SW480 cells, despite best efforts within the time available. I would hope that further research outwith the scope of the work for this thesis could confirm or refute the hypothesis that IκBα is phosphorylated on tyrosine 42 in response to non-aspirin NSAIDs. This is especially the case given the exciting experimental observations described below.

### 4.2.2 Inhibition of NSAID Effects on NF-κB Signaling by Chemical Tyrosine Kinase Inhibitors

To investigate the hypothesis generated by the literature search that tyrosine phosphorylation could be involved in the NF-κB response to non-aspirin NSAIDs, the effects of tyrosine kinase inhibitors on NSAID-mediated activation of the NF-κB pathway were studied. In the first instance, the broad spectrum tyrosine kinase inhibitor, genistein, was employed. Genistein did not block NSAID-induced nuclear or nucleolar translocation of RelA in SW480 cells, as defined by immunocytochemistry (Figure 4.4). Genistein was noted to be toxic to SW480 cells,

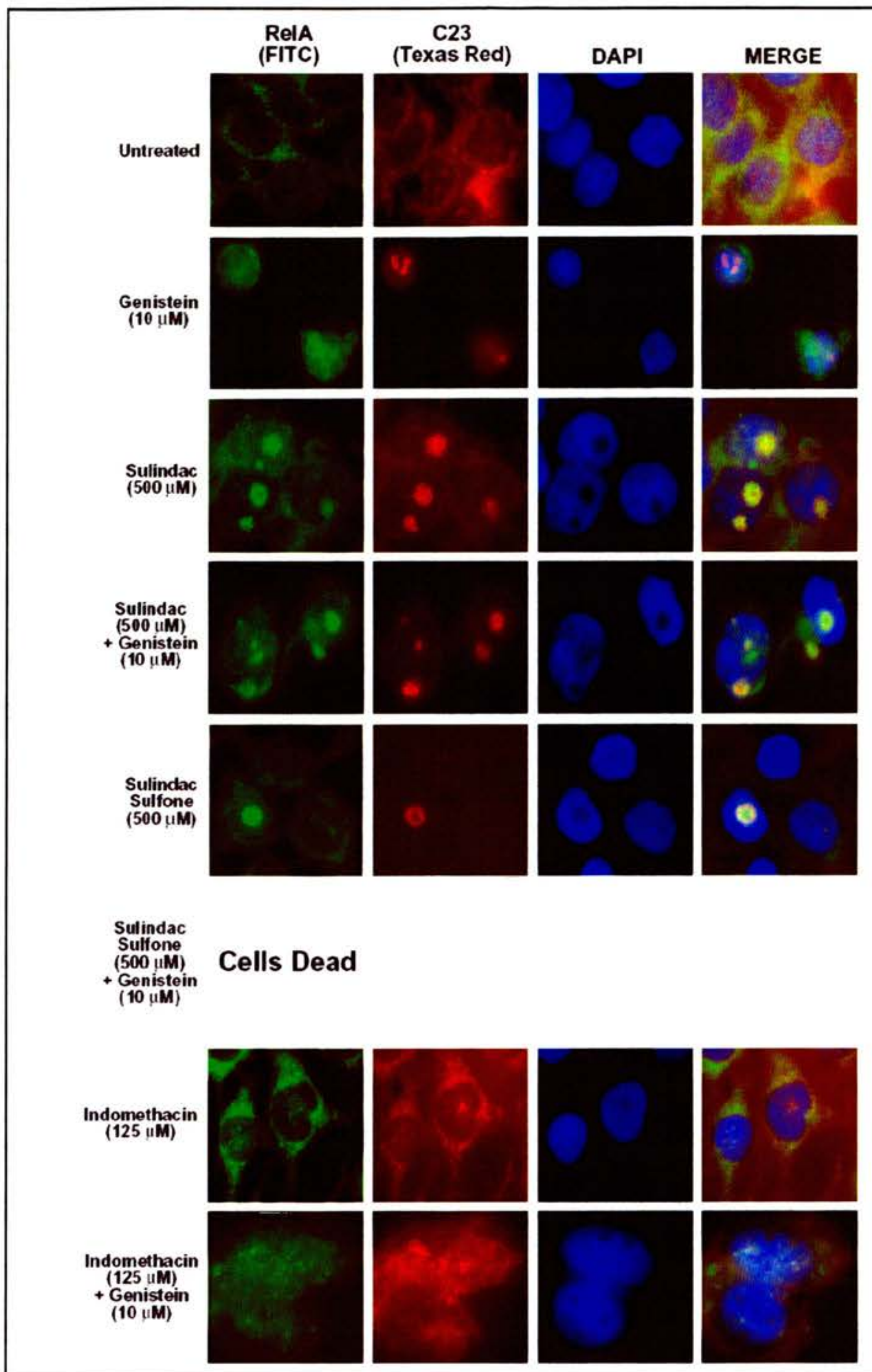



**Figure 4.4 – Genistein does not Block NSAID-induced Nuclear and Nucleolar Translocation of RelA.** In three independent experiments, SW480 cells were pre-treated with Genistein (10 μM) for 1 hr prior to overnight treatment (16 hrs) with Sulindac (500 μM), Sulindac Sulfone (500 μM), Indomethacin (125 μM) or untreated (control). Representative micrographs (63 x) illustrating localisation of RelA and the nucleolar protein, C23, are shown.

particularly when cells were also treated with sulindac sulfone. This line of investigation using a non-specific tyrosine kinase inhibitor was therefore essentially unhelpful. Hence, attention was turned to a candidate tyrosine kinase of IκBα. Although the kinase responsible for tyrosine phosphorylation of IκBα has not been defined to date, c-Src is a particularly good candidate (see 4.1). Therefore, the next focus of investigation was to determine the effects of inhibition of c-Src, using chemical and genetic inhibition, on NSAID-mediated activation of NF-κB.

### 4.2.3 c-Src Inhibitor PP2 Blocks Nuclear Translocation of RelA after NSAID Treatment

Cells were exposed to PP2, a c-Src inhibitor, and to NSAIDs in order to determine whether NSAID-induced nuclear and nucleolar translocation of RelA could be blocked. Immunocytochemistry indicated that in untreated cells and cells that were treated with the PP2 inhibitor alone, RelA was predominantly cytoplasmic (Figure 4.5). In contrast, cells treated with sulindac, sulindac sulfone or indomethacin alone exhibited nucleolar RelA, as described in Chapter 3. Strikingly, the PP2 inhibitor completely blocked the nuclear translocation and nucleolar sequestration of RelA in response to the non-aspirin NSAIDs but did not inhibit the effects of aspirin on NF-κB activation. This is highly suggestive that aspirin and non-aspirin NSAIDs activate NF-κB signaling by alternative pathways.

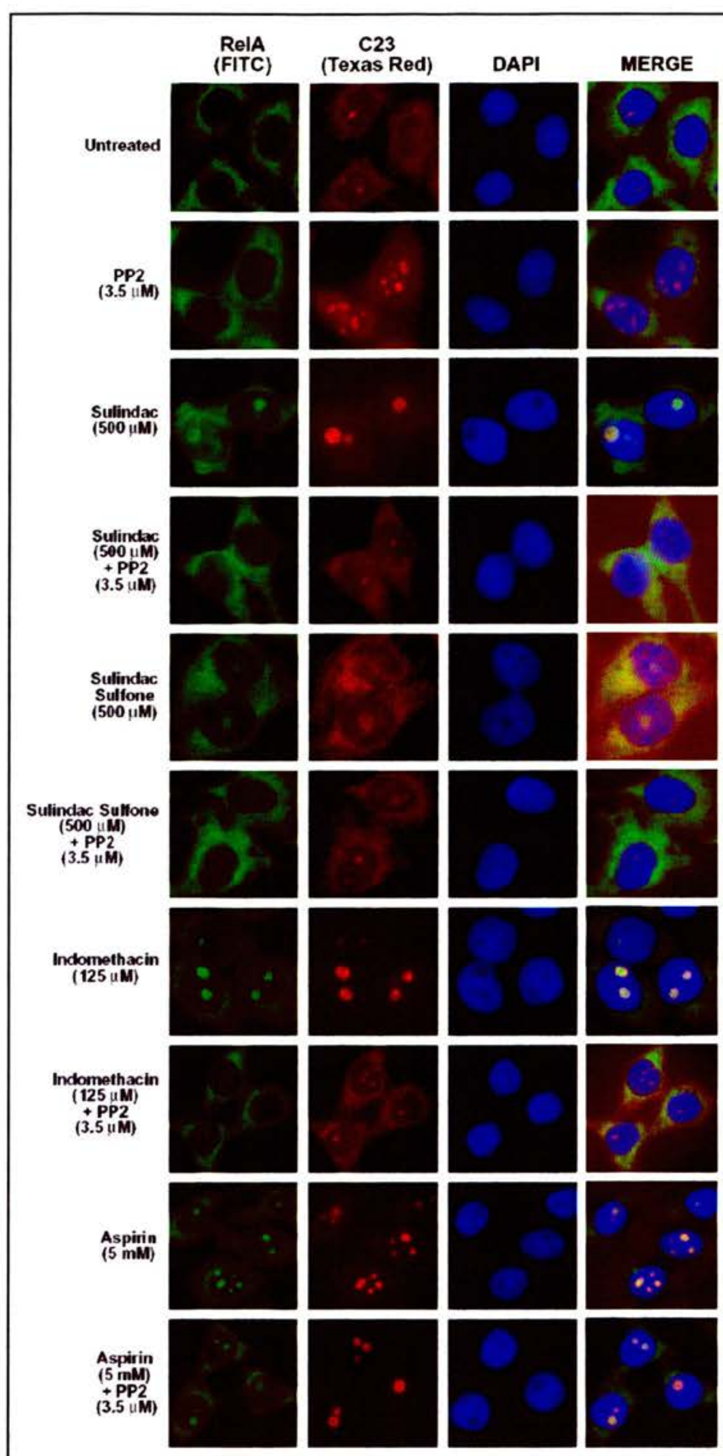



**Figure 4.5 – c-Src Inhibitor PP2 Blocks Nucleolar Localisation of RelA After NSAID Treatment.** In three independent experiments, SW480 cells were pre-treated with c-Src inhibitor PP2 (3.5 μM) for 1 hr prior to overnight treatment (16 hrs) with Sulindac (500 μM), Sulindac Sulfone (500 μM), Indomethacin (125 μM), Aspirin (5 mM) or untreated (control). Representative micrographs (63 x) illustrating localisation of RelA and the nucleolar protein, C23, are shown.

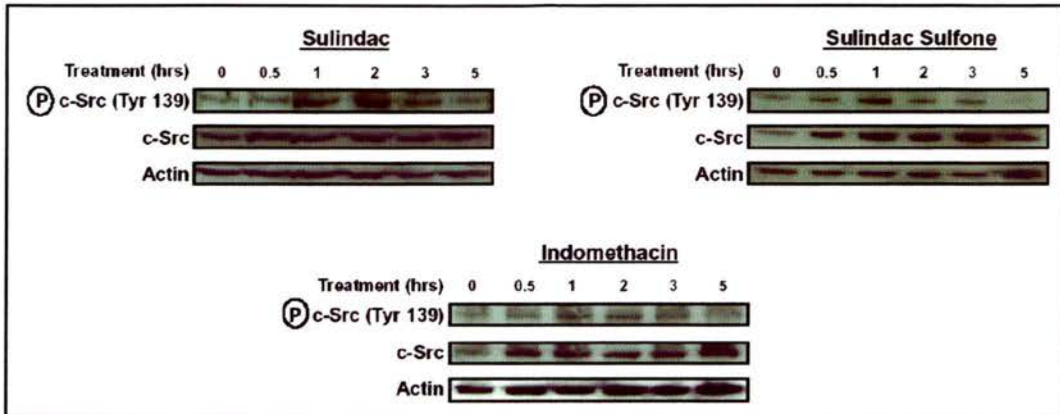
### 4.2.4 c-Src is Phosphorylated and Activated after NSAID Treatment

To further determine the role of c-Src in NSAID effects on the NF-κB pathway, the activation status of this kinase in response to sulindac, sulindac sulfone and indomethacin was examined. Since phosphorylation of c-Src on tyrosines 139 and 416 renders the protein catalytically active (Boggon and Eck, 2004), phospho-specific antibodies to tyrosine 139 and 416 of c-Src were used to determine whether NSAIDs modulated the activity of this kinase. Using Western blot analysis in kinetic studies, an increase in Tyr 139 (Figure 4.6A) and Tyr 416 (Figure 4.6B) phosphorylated c-Src was observed at 1-2 hrs after treatment with sulindac, sulindac sulfone and indomethacin, which then decreased again after 5 hrs. Furthermore, NSAIDs had minimal effects on the level of endogenous c-Src. This data strongly suggests that c-Src is activated by this panel of non-aspirin NSAIDs.

### 4.2.5 SW480-SrcKD are Resistant to NSAID-induced Apoptosis

When combined with NSAIDs, PP2 has similar toxic effects to tyrosine kinase inhibitors as a class on SW480 cells and so it was difficult to use this agent to determine the role of c-Src in NSAID-mediated apoptosis. Therefore, SW480 cells stably transfected with a kinase dead form of c-Src (SW480-SrcKD) were obtained (kind gift from Prof. M Frame, Cancer Research UK Beatson Laboratories) along with control cells stably transfected with the pBpuro empty vector alone (SW480-

**A**

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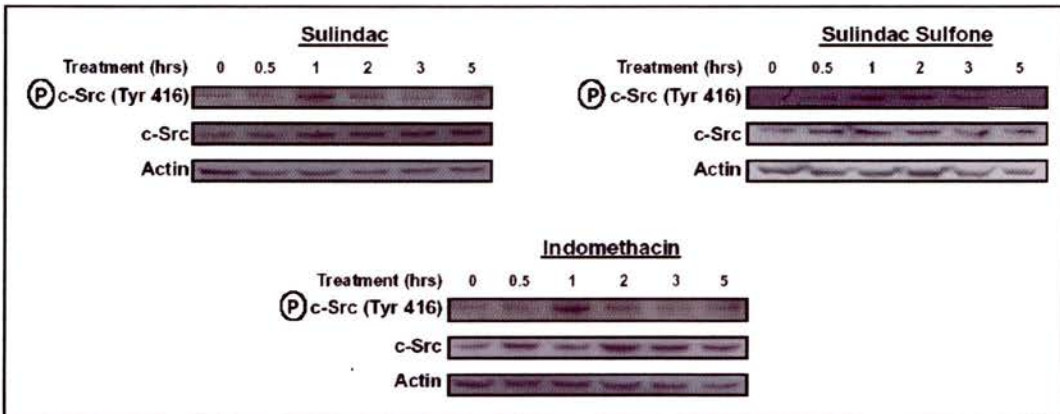



**Figure 4.6 – NSAID Treatment Causes Activation of c-Src.** **(A)** SW480 cells were treated, in a minimum of three independent experiments, for 0-5 hrs with Sulindac (500 μM), Sulindac Sulfone (500 μM) or Indomethacin (250 μM), then cytoplasmic extracts (25 μg) resolved by SDS PAGE on 8% polyacrylamide gels and anti-phospho-c-Src (Tyr 139) Western blot carried out. Blots were stripped and re-probed for endogenous c-Src and subsequently actin (loading control). Representative blots are shown. **(B)** In three independent experiments, SW480 cells were treated for 0-5 hrs with Sulindac (500 μM) Sulindac Sulfone (500 μM) or Indomethacin (250 μM), then cytoplasmic extracts (25 μg) resolved by SDS PAGE on 8% polyacrylamide gels and anti-phospho-c-Src (Tyr 416) Western blot carried out. Blots were stripped and re-probed for endogenous c-Src and subsequently actin (loading control). Representative blots are shown.

pBpuro). SW480-pBpuro and SW480-SrcKD cells were treated overnight with sulindac, sulindac sulfone and indomethacin and AnnexinV apoptosis assay used to determine the percentage apoptosis (Figure 4.7). The SW480-pBpuro cell line showed a dose-responsive increase in apoptosis with all the NSAIDs. This effect of NSAID treatment was almost completely abrogated in the SW480-SrcKD cell line, providing compelling evidence for a role of c-Src in NSAID-induced apoptosis.

### 4.2.6 SW480-SrcKD are Resistant to NSAID-induced Repression of Basal NF-κB Activity

Since previous studies from the host laboratory indicated that NSAID-mediated activation of the NF-κB pathway causes a decrease in NF-κB-driven transcription (Stark *et al.*, 2001; Stark and Dunlop, 2005), the effect of ablating c-Src kinase activity in the SW480-SrcKD cells was assessed with respect to the NSAID response described above. SW480-pBpuro and SW480-SrcKD cells were transiently transfected with the 3x κB ConA-Luc NF-κB dependent luciferase reporter plasmid along with the pCMVβ control plasmid. Luciferase assays demonstrated repression of NF-κB activity in response to NSAIDs in the control cell line (Figure 4.8) and this response was similar to that which was previously observed in parental SW480 cells (Figure 3.4). In contrast, repression of NF-κB activity by NSAIDs is abrogated in the SW480-SrcKD cells, indicating that c-Src activity is required for NSAID induced down regulation of NF-κB-driven transcrition.

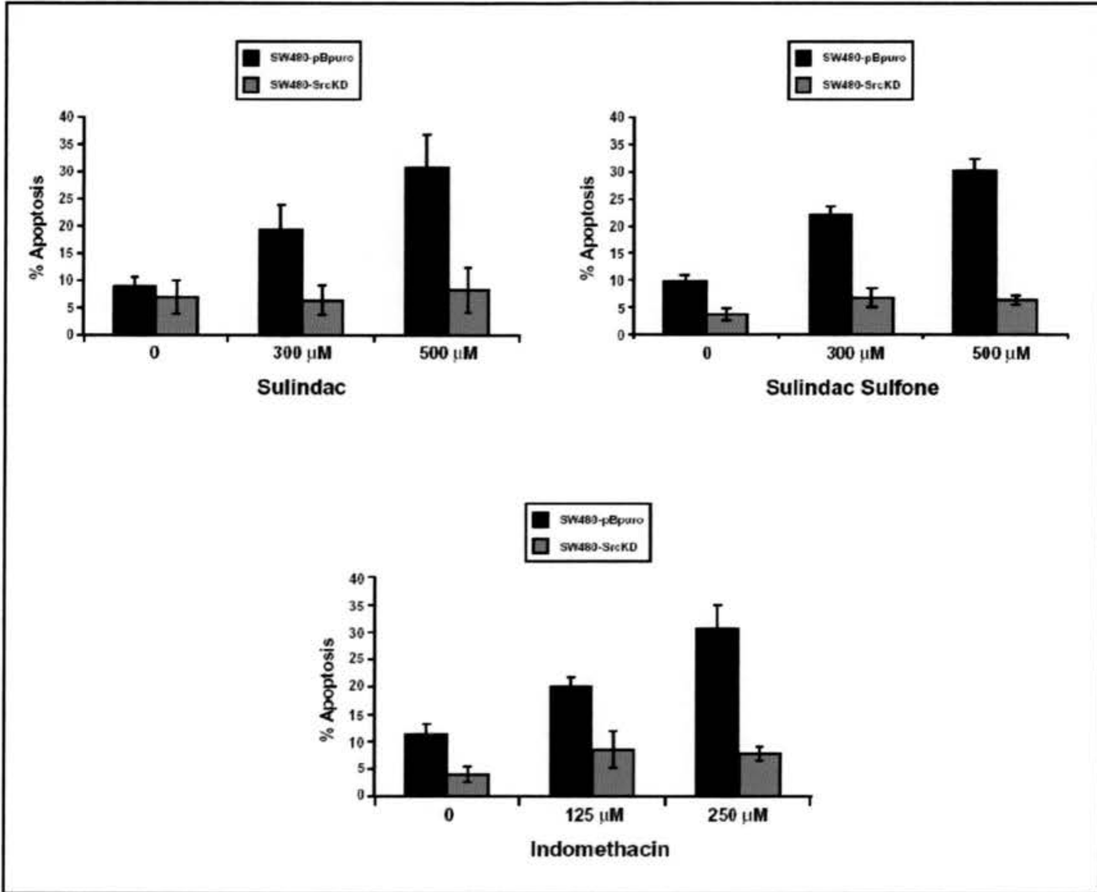



**Figure 4.7 – SW480 Cells Overexpressing Kinase Dead c-Src (SrcKD) are Resistant to NSAID-induced Apoptosis.** SW480-pBpuro and SW480-SrcKD cells were treated overnight (16 hrs) with Sulindac (0, 300 µM, 500 µM), Sulindac Sulfone (0, 300 µM, 500 µM) or Indomethacin (0, 125 µM, 250 µM). The percentage of apoptotic cells was determined using fluorescence microscopy, in a minimum of 200 cells from multiple fields of view, by Annexin V-FITC binding to externalised phosphatidylserine. Results presented are the mean of three independent experiments (+/- standard deviation).

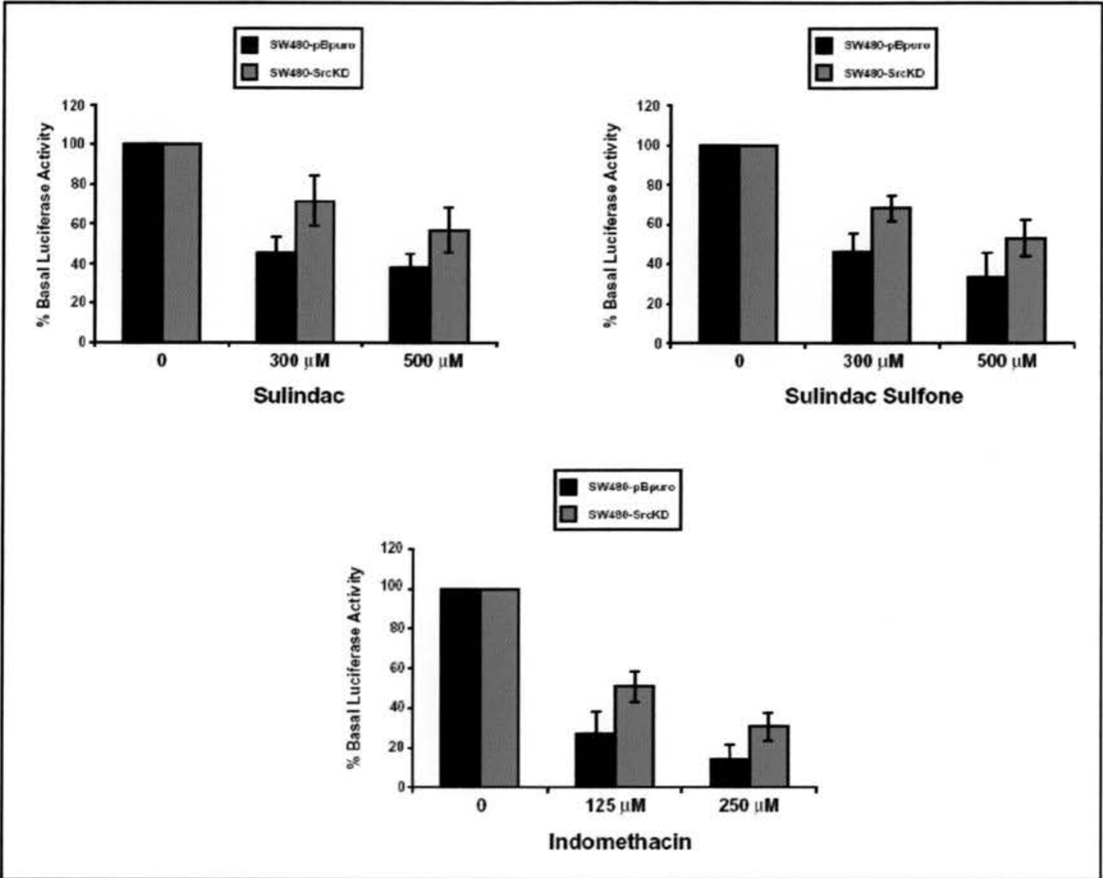



**Figure 4.8 – SW480 Cells Expressing Kinase Dead c-Src are Resistant to NSAID-induced Repression of NF-κB Transcriptional Activity.** SW480-pBpuro and SW480-SrcKD cells were transiently transfected with 3x κB ConA-Luc and pCMVβ, then treated overnight (16 hrs) with Sulindac (0, 300 μM, 500 μM) Sulindac Sulfone (0, 300 μM, 500 μM) or Indomethacin (0, 125 μM, 250 μM). Luciferase assays were subsequently carried out to measure NF-κB transcriptional activity and β-galactosidase assays were used to normalise these results for transfection efficiency. % transcriptional activity was calculated relative to non-treated controls. Results presented are the mean of three independent experiments (+/- standard deviation).

### 4.2.7 Investigation of Phosphorylation of IκBα by c-Src

The data presented above provides evidence that c-Src is activated in response to the panel of non-aspirin NSAIDs. Furthermore, inhibition of c-Src kinase blocks NSAID-mediated activation of the NF-κB pathway, downregulation of NF-κB driven transcription and apoptosis. In light of this evidence and in order to further investigate the hypothesis that tyrosine phosphorylation of IκBα occurs in response to treatment with the panel of non-aspirin NSAIDs, the next focus of investigation was to determine whether c-Src could directly phosphorylate IκBα after NSAID treatment.

As a first step, SW480 cells were treated for 3 hrs with sulindac or 1 hr with TNFα/LPS/EtOH and immunoprecipitation followed by Western blot analysis performed to check that c-Src immunoprecipitations were robust before undertaking kinase assays. Figure 4.9 demonstrates that c-Src is specifically immunoprecipitated from the extracts in an antibody-dependent manner.

Cold *in vitro* kinase assays, performed as described in Chapter 2, using c-Src immunoprecipitated from SW480 cells treated with either sulindac (500 μM for 5 hrs) or pervanadate (100 μM for 5 hrs) and recombinant IκBα as a substrate, indicated that under both these conditions, c-Src does *not* directly phosporylate IκBα (Figure 4.10).

Next, to confirm that active c-Src was being pulled out of treated cells, the assay was repeated using a short synthetic peptide from $p34^{cdc2}$, which can act as a substrate for c-Src (Cheng *et al.*, 1992). TNFα /LPS/EtOH was used a positive

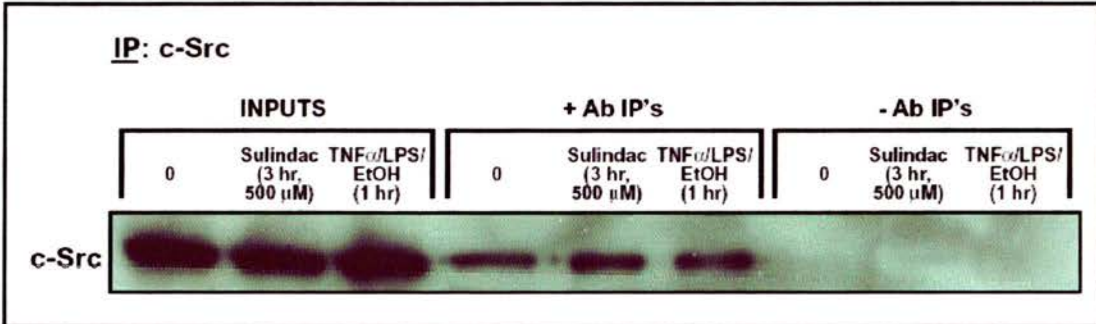



**Figure 4.9 – c-Src is Specifically Immunoprecipitated After Sulindac and TNFα/LPS/EtOH Treatment.** SW480 cells were treated, in three independent experiments, for 3 hrs with Sulindac (0, 500 μM) or 1 hr with TNFα/LPS/EtOH [(1 ng/ml)/(1 μg/ml)/(1 mM)] then whole cell extracts (500 μg) immunoprecipitated (IP) using anti-c-Src (rabbit) antibody (+ Ab) or no antibody (- Ab) as a control. Inputs (30 μg) and immunoprecipitates were resolved by SDS PAGE on 8% polyacrylamide gels and Western blot analysis, using anti-c-Src (mouse) antibody, carried out. Representative blot is shown.

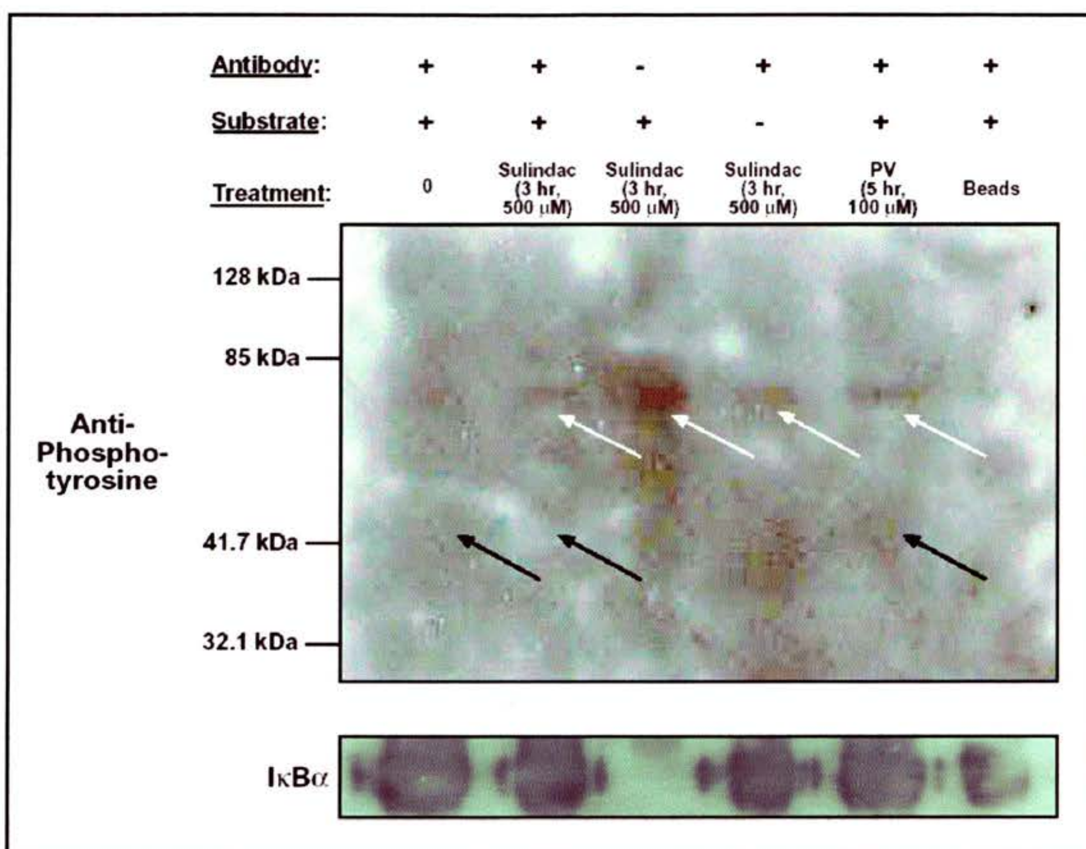



**Figure 4.10 – IκBα is not Phosphorylated by Immunoprecipitated c-Src After Sulindac or Pervanadate Treatment.** In three independent experiments, SW480 cells were treated for 3 hrs with Sulindac (0, 500 μM) or 5 hrs with Pervanadate (PV) (100 μM) then whole cell extracts (500 μg) immunoprecipitated using anti-c-Src (rabbit) antibody. Cold *in vitro* kinase assay was subsequently carried out using immunoprecipitated c-Src and recombinant IκBα as a substrate by a standard method (see Chapter 2). Kinase assay reactions were then resolved by SDS PAGE on 10% polyacrylamide gels and Western blot analysis, using an anti-phospho-tyrosine antibody, carried out. Blots were stripped and re-probed with rabbit polyclonal anti-IκBα antibody. Representative blots are shown. White arrows indicate faint c-Src band; black arrows indicate position on blot where band for IκBα would have been if it was phosphorylated.

control in this instance as it has been shown that c-Src is strongly activated in response to the combination of these agents (Kuo *et al.*, 1997). Phosphorylation of the synthetic peptide and recombinant IκBα was detected using a scintillation counter (see Chapter 2). Table 4.1 indicates that this method of detection was inconsistent, for instance, the moles of phosphate transferred in the no-NSAID control in experiment was 1.18 in the first experiment, 43.97 in the second experiment and 7.30 in the third experiment, and so the results of these experiments were therefore inconclusive.

The above description was disappointing in terms of its lack of provision of a definitive answer as to whether or not IκBα is phosphorylated on tyrosine 42 by c-Src in SW480 cells in response to sulindac, again despite best efforts in the allotted time available. It should be noted that similar technical difficulties to those outlined above, that is problems with protein transfer and/or the anti-phospho-tyrosine antibody, were experienced when trying to detect tyrosine phosphorylated IκBα as the end point of the cold kinase assay procedure. Therefore, blots of these kinase assays were very difficult to read and interpret. In addition to the lack of a robust method for detection of tyrosine phosphorylated IκBα, another major problem was the lack of a good positive control stimuli for this event in SW480 cells. I would hope that further research outwith the scope of the work for this thesis could confirm or refute the hypothesis that IκBα is phosphorylated on tyrosine 42 by c-Src in response to non-aspirin NSAIDs.

| Sample | Substrate | Antibody Used For IP | Moles Phosphate Transferred (Expt. 1) | Moles Phosphate Transferred (Expt. 2) | Moles Phosphate Transferred (Expt. 3) |
|---|---|---|---|---|---|
| No NSAID | Src | Src | 1.18 | 43.97 | 7.30 |
| TNFα/LPS/EtOH [1 hr, (1 ng/ml)/(1 μg/ml)/(1 mM)] | - | Src | - | - | - |
| TNFα/LPS/EtOH [1 hr, (1 ng/ml)/(1 μg/ml)/(1 mM)] | Src | - | 5.25 | 1.15 | 87.36 |
| TNFα/LPS/EtOH [1 hr, (1 ng/ml)/(1 μg/ml)/(1 mM)] | Src | Src | 3.13 | 0.27 | 1.56 |
| Sulindac (3 hrs, 500 μM) | Src | Src | 0.83 | 1.78 | -1.21 |
| No NSAID | IκBα | Src | 0.34 | 2.61 | 5.69 |
| TNFα/LPS/EtOH [1 hr, (1 ng/ml)/(1 μg/ml)/(1 mM)] | IκBα | Src | 0.04 | 3.16 | -1.35 |
| Sulindac (3 hrs, 500 μM) | IκBα | Src | 0.57 | 0.97 | 0.88 |

**Table 4.1 – Results of c-Src Kinase Assays by Scintillation Counting.** SW480 cells were treated for 3 hrs with Sulindac (0, 500 μM) or for 1 hr with TNFα/LPS/EtOH [(1 ng/ml)/(1 μg/ml)/(1 mM)] then whole cell extracts (500 μg) immunoprecipitated (IP) using anti-c-Src antibody. Hot *in vitro* kinase assays were subsequently carried out by a standard method (see Chapter 2) using immunoprecipitated c-Src with either recombinant IκBα as a substrate or a short synthetic peptide (Src substrate), as a positive control. Phosphorylation of recombinant IκBα and the synthetic Src substrate was detected by scintillation counting. The results in the table represent the findings of three independent experiments (Expt.).

## 4.3 Discussion

Activation of the NF-κB pathway by inflammatory cytokines has been been extensively investigated and characterised. It involves activation of NIK, IKK and ubiquitin-mediated proteolysis of IκBα (described in Chapter 1). However, the pathways by which other agents exert their effects on the NF-κB pathway remain poorly understood, particularly agents inducing cellular stress. Agents such as pervanadate (Imbert *et al.*, 1996; Singh *et al.*, 1996), hypoxia/reoxygenation (Imbert *et al.*, 1996) and NGF (Bui *et al.*, 2001) have been shown to cause nuclear translocation of NF-κB in the absence of IκBα degradation. Moreover, phosphorylation of IκBα on tyrosine 42 is implicated in this mechanism of activation of NF-κB in response to these agents. Since it was observed in the previous chapter that non-aspirin NSAIDs activate the NF-κB pathway in the absence of IκBα degradation, one possibility is that these agents cause tyrosine phosphorylation of IκBα. Hence the major objective of this chapter was to investigate whether tyrosine 42 phosphorylation of IκBα occurred in response to NSAIDs and whether this mechanism explained the effects presented in Chapter 3.

Initial experiments aimed at blocking the effects of NSAIDs on NF-κB using genistein, a non-specific tyrosine kinase inhibitor, proved that it was too toxic to use in such experiments (Figure 4.4). A more specific, less toxic inhibitor was therefore required. Of the candidate tyrosine kinases for IκBα outlined in the introduction, only c-Abl (Kawai *et al.*, 2002), $p56^{lck}$ (Mahabeleshwar and Kundu, 2003) and c-Src (Abu-Amer *et al.*, 1998; Fan *et al.*, 2003) have been shown to directly phosphorylate

IκBα. c-Src was further investigated because it has been shown to play a role in colorectal cancer progression and there is evidence that it phosphorylates IκBα.

While accepting that it is far from conclusive, the data presented in this chapter is commensurate with the notion that c-Src modulates the NF-κB pathway via direct or indirect tyrosine phosphorylation of IκBα in response to NSAIDs. The evidence to support this hypothesis is based on the following observations: **1**. PP2, a c-Src inhibitor, blocks nucleolar translocation of RelA in response to sulindac, sulindac sulfone and indomethacin (Figure 4.5). **2**. c-Src is phosphorylated on tyrosines 139 and 416 at 1-2 hrs treatment with the panel of non-aspirin NSAIDs (Figure 4.6), indicating the kinase is catalytically active. **3**. SW480 cells stably transfected with a kinase dead c-Src construct are resistant to NSAID-induced apoptosis and repression of NF-κB activity (Figures 4.7 and 4.8). This is the first study to indicate a role for c-Src in the mechanism of action of NSAIDs and in particular, it implicates c-Src in the pathway to activation of NF-κB signaling and apoptosis in response to sulindac, sulindac sulfone and indomethacin.

It is noteworthy that PP2 does not inhibit c-Src activity by competing with ATP for the active site on c-Src, but instead acts as a 'mixed competitive' inhibitor with the substrate (Karni *et al*., 2003). Furthermore, PP2 has been shown to inhibit the activity of other Src family kinase members (Hanke *et al*., 1996), indicating that it is not wholly specific to c-Src. It would therefore be important to establish that PP2 selectively inhibits c-Src activation in response to NSAIDs. Further work is also required to confirm that the SW480-SrcKD cell line is indeed kinase dead for c-Src activity, as it was not possible to complete this work during the period of research. Moreover, it will be important to use SW480 cells stably transfected with wild-type

c-Src to demonstrate that the observed effects are not due to overexpression (SW480 cells stably transfected with wild-type c-Src were not available from the Frame lab and it was not possible to generate these cells in the time available for this research). When reviewing these experiments, it would have been a good idea to quantitate the PP2 data (Figure 4.5) to allow the use of statistics to demonstrate an effect. It would also have been of benefit to employ statistics on the apoptosis and repression data for the SW480-pBpuro and SW480-SrcKD cell lines (Figures 4.7 and 4.8) and to use controls that could cause apoptosis and repression in the SW480-SrcKD cell line in those experiments respectively in order to demonstrate a specific effect.

Tyrosine phosphorylation of IκBα is known to occur within 30-60 mins following exposure of cells to treatment with pervanadate or hypoxia/reoxygenation (Imbert *et al.*, 1996). Given that c-Src is activated at 1-2 hrs of NSAID treatment, it is plausible that c-Src directly phosphorylates IκBα in response to these agents. However, it is also possible that c-Src activates another tyrosine kinase or a cascade of kinases culminating in phosphorylation of IκBα. It is of particular interest that several studies provide evidence for a role for PI 3-kinase in NF-κB activation by the tyrosine phosphorylation-dependent pathway. The regulatory subunit of PI 3-kinase, p85α, has been shown to directly interact with v-Src, the gene product of the v-*src* oncogene of Rous sarcoma virus (Haefner *et al.*, 1995). Another report (Béraud *et al.*, 1999) demonstrated that p85α can associate with tyrosine phosphorylated IκBα in response to pervanadate treatment via its Src homology domains. A more recent study (Kang *et al.*, 2003) has also documented interaction of tyrosine phosphorylated IκBα with p85α, but in response to silica. It is also worth mentioning that focal

adhesion kinase (FAK), a primary target of c-Src (Martin, 2003; Frame, 2004), has been shown to phosphorylate PI 3-kinase (Cary and Guan, 1999).

Although the favoured hypothesis from the data presented is that non-aspirin NSAIDs cause c-Src-mediated activation of NF-κB through direct or indirect tyrosine phosphorylation of IκBα, it should be pointed out that tyrosine phosphorylated IκBα was not detected after NSAID treatment, despite using several different approaches (see 4.2.1) and that kinase assays used to investigate phosphorylation of IκBα by c-Src were inconclusive (see 4.2.7). Indeed, when re-evaluating these data, it may have been better to have considered studying very early time points, such as 15 min to 1 hr, in kinase assays and when trying to detect tyrosine phosphorylated IκBα in response to NSAIDs and/or the positive control, pervanadate, as tyrosine phosphorylation of IκBα could be a very rapid and transient event. It should also be noted that overexpression of IκBα did not prove to be all that successful in Figure 4.3, as the band for PK-tagged IκBα was very faint. Moreover, it might have been a better idea to perform immunoprecipitation for the PK tag in that experiment. Taking these points of consideration into account, future work beyond the scope of this thesis may therefore provide clearer results. However, it is also conceivable that tyrosine phosphorylation of IκBα is not responsible for the observed activation of NF-κB by non-aspirin NSAIDs.

One possibility is that c-Src activates the NF-κB pathway by an indirect mechanism. Previous studies have shown that c-Src can activate NF-κB via tyrosine phosphorylation of the IKK complex (Huang *et al.*, 2003a and 2003b). It has also recently been reported that c-Src acts synergistically with IL-1 in inducing NF-κB activation via interaction with IKKγ, and that this is independent of the tyrosine

kinase activity of c-Src (Funakoshi-Tago *et al.*, 2005). However, in those studies, activation of IKK was found induce serine phosphorylation and degradation of IκBα and so it is unlikely that c-Src mediates activaton of IKK in response to NSAIDs.

Another possibility could be that NSAIDs activate NF-κB, through c-Src, by directly targeting RelA. There is substantial evidence that the nuclear activity of NF-κB can be modulated by direct phosphorylation of RelA (see Chapter 1). Interestingly, a recent study (Kang *et al.*, 2006) demonstrated that c-Src mediates crystalline silica-induced NF-κB activation in RAW 264.7 macrophages. Moreover, in that study, silica was found to induce tyrosine phosphorylation of both IκBα and RelA, events that could be blocked by selective Src tyrosine kinase inhibitors. It has also previously been reported that treatment of human umbilical vein endothelial cells with resveratrol, a naturally occurring anti-inflammatory agent, increases tyrosine phosphorylation of both IκBα and RelA in the presence or absence of a short burst (30 min) of treatment with TNFα (Pellegatta *et al.*, 2003), leading the authors to postulate that resveratrol exerts its effects through direct modulation of NF-κB transcriptional activity. In relation to the data presented here, it is therefore conceivable that c-Src could directly phosphorylate RelA on tyrosine in response to NSAIDs, thus allowing activation of NF-κB in an IκBα-independent manner.

In this study, sulindac, sulindac sulfone and indomethacin exposure of SW480 cells was followed by c-Src phosphorylation on residues Tyr 139 and Tyr 416 (Figure 4.6). Hence, it was concluded that these agents activate the kinase. Several mechanisms for regulation of c-Src have been proposed [reviewed in (Boggon and Eck, 2004; Frame, 2002; Bjorge *et al.*, 2000)]. Src family kinases share a conserved domain organisation, comprising a myristoylated N-terminal segment,

termed the SH4 membrane targeting region, which is followed by a 'unique' domain that differs among family members (Boggon and Eck, 2004). This in turn is followed by consecutive SH3, SH2, linker and tyrosine kinase (SH1) domains, and a short C-terminal tail (Ishizawar and Parsons, 2004). Comparable to most protein kinases, c-Src requires autophosphorylation within a segment of its kinase domain, termed the activation loop, for optimal catalytic activity (Boggon and Eck, 2004). In c-Src, this autophosphorylation site is Tyrosine 416. Autophosphorylation of c-Src is negatively regulated by phosphorylation of a carboxy-terminal tyrosine residue (Tyrosine 527), termed the autoinhibitory phosphorylation site, by related tyrosine kinases, Csk (c-Src kinase) and CHK (CSK homologous kinase), which leads to binding of this region of the protein to the SH2 domain and a 'closed' or inactive conformation of c-Src (Playford and Schaller, 2004). Protein tyrosine phosphatases which dephosphorylate Tyrosine 527, for example PTP-α, or reduced Csk activity, can release the negative inhibition of phosphorylation of this residue on the complex. Displacement of intramolecular SH-binding interactions can also cause c-Src to adopt an active conformation (Alper and Bowden, 2005). For example, FAK is a c-Src effector that can bind to the SH domains and lead to c-Src kinase activation (Frame, 2002). It would therefore be appealing to further examine c-Src kinase activity, particularly the phosphorylation status at residue Tyr 527, to confirm the activation status of c-Src in response to NSAIDs.

A key question arising from the data in this study is how c-Src itself is targeted in response to NSAID treatment. Previous studies have shown that c-Src can be activated by extracellular matrix (ECM) contact that is mediated by integrin receptors (Jones *et al.*, 2000; Westhoff *et al.*, 2004). This is of particular interest as

aspirin (Jiang *et al.*, 2001) and the selective COX-2 inhibitor, JTE-522 (Yazawa *et al.*, 2005) have been shown to target the extracellular matrix. Indomethacin has also very recently been reported to enhance the expression of E-cadherin, a key cell adhesion molecule (Kapitanović *et al.*, 2006). Integrin adhesion to extracellular matrix proteins is essential to protect adherent cells from a form of detachment-induced apoptosis called anoikis (Meredith, Jr. *et al.*, 1993) and c-Src activation has been shown to regulate anoikis in human colon tumour cell lines (Windham *et al.*, 2002). Interestingly, it was recently documented that integrin $\alpha_v\beta_3$ ligation in multiple cell types induced activation of NF-κB and that inhibition of the activity of the family of Src kinases blocked this activation, in particular c-Src (Courter *et al.*, 2005). Therefore, it is possible that NSAIDs activate c-Src through integrin signaling resulting from loss of cell adhesion. Alternatively, c-Src could be activated via promotion of protein tyrosine phosphatase activity, inhibition of Csk activity or activation of a c-Src effector, such as FAK. Future research is required to establish the exact mechanism of c-Src activation in response to NSAIDs. In particular, it would be of interest to use siRNA directed against c-Src to investigate the upstream pathway to actvation of c-Src by NSAIDs and the role of c-Src in activation of the NF-κB pathway and apoptosis induced by NSAIDs

Collectively, the data presented in this chapter and the previous chapter establishes a novel mechanism for NSAID-induced apoptosis of colorectal cancer cells, involving activation of c-Src kinase. The results are preliminary with respect to the mechanism by which the observations come about but nonetheless, the data suggest c-Src is implicated in the mechanism of action of NSAIDs.

# Chapter 5 – Regulation of Nuclear NF-κB Function by β-catenin

## 5.1 Introduction

The host laboratory has previously demonstrated nucleolar localisation of RelA in response to aspirin treatment (Stark and Dunlop, 2005). In that study, compartmentalisation of RelA in the nucleolus was found to be stimulus specific. Whereas the classical NF-κB activators, TNF and TRAIL, caused RelA to be concentrated in the nucleoplasm, the pro-apoptotic stimuli serum withdrawal and UV-C radiation resulted in accumulation of RelA in the nucleolus. The observation that both cyclohexamide and actinomycin D prevented RelA translocation to the nucleolus indicated that de-novo protein synthesis was required for nucleolar sequestration of RelA. Significantly, localisation of RelA in the nucleolus required translocation from the cytoplasm to the nucleus and was causally involved with repression of NF-κB transcriptional activity and consequently, apoptosis (Stark and Dunlop, 2005).

In chapter 3, nucleolar localisation of RelA is shown following exposure to sulindac, sulindac sulfone and indomethacin and in a similar manner to aspirin, these events were associated with transcriptional repression of NF-κB activity and apoptosis. Collectively, these data confirm the importance of nucleolar translocation of RelA in NSAID-induced apoptosis. However, the precise mechanisms regulating nucleolar sequestration of RelA and the role of RelA in the nucleolus are unknown.

For that reason, it is critical to identify the factors that regulate nuclear distribution of RelA because if these pathways were understood, one could envisage the development of small molecules that target RelA to this nuclear compartment to induce apoptosis of colorectal cancer cells. Hence, the major objective of the following two chapters was to investigate the mechanisms involved in nucleolar localisation of RelA

One potential candidate for regulating nuclear NF-κB function in response to NSAIDs is β-catenin. It is well established that one of the initial stages of colorectal carcinogenesis is mutation of *APC*, which affects Wnt signaling and thus causes dysregulation of β-catenin levels (Fearon and Vogelstein, 1990). This ultimately results in aberrant β-catenin/TCF-mediated transcriptional activity and hence inappropriate expression of target genes, such as *Cyclin D1* (Giles *et al.*, 2003), eventually leading to tumorigenesis. β-catenin is a particularly good candidate for several reasons. Firstly, several studies have shown that NSAIDs modulate β-catenin. In particular, NSAIDs have been shown to induce degradation of β-catenin (Rice *et al.*, 2003); alter expression of β-catenin (Kapitanović *et al.*, 2006); target nuclear accumulation of β-catenin (Thompson *et al.*, 2000; Boon *et al.*, 2004); downregulate β-catenin/TCF-driven transcriptional activity (Dihlmann *et al.*, 2001 and 2003; Gardner *et al.*, 2004; Veeramachaneni *et al.*, 2003) and alter expression of β-catenin/TCF target genes (Hawcroft *et al.*, 2002; Li *et al.*, 2002). Secondly, the β-catenin and NF-κB pathways have similar properties. The kinases, GSK-3β and IKK, are common to both signaling pathways (Hoeflich *et al.*, 2000; Lamberti *et al.*, 2001); both β-catenin and IκB interact with the same E3 ubiquitin ligase (Amit and Ben Neriah, 2003); and NF-κB and β-catenin regulate a similar panel of genes (Pahl,

1999; Polakis, 2000). Finally, β-catenin interacts with NF-κB. It has previously been reported that β-catenin can physically interact with RelA and that this results in reduced NF-κB DNA binding (Deng *et al.*, 2002). A more recent study by the same group established that APC/GSK-3β, through β-catenin, can cross-regulate the NF-κB pathway (Deng *et al.*, 2004). It has also been reported that RelA can suppress the Wnt/β-catenin pathway (Masui *et al.*, 2002). Interestingly, a recent study (Cho *et al.*, 2005) demonstrated that the NSAID diclofenac attenuates Wnt/β-catenin signaling in colon cancer cells by activation of NF-κB.

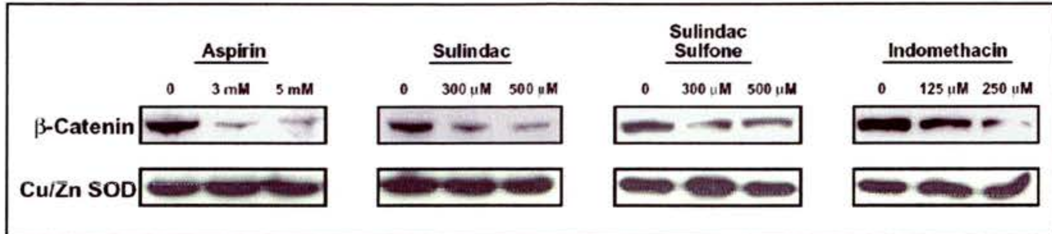
In light of previous evidence, the specific aim of this chapter was therefore to investigate the potential relationship between NSAID effects on NF-κB signaling and the function and nuclear distribution of β-catenin.

## 5.2 Results

### 5.2.1 NSAIDs Induce Proteasome-mediated Degradation of Cytoplasmic β-catenin

In order to investigate NSAID effects on β-catenin function, Western blot analysis was initially used to look at cytoplasmic β-catenin levels in SW480 colorectal cancer cells after treatment with sulindac, sulindac sulfone, indomethacin and aspirin. From the results in Figure 5.1A, cytoplasmic β-catenin levels decreased in response to treatment with all the NSAIDs used in a dose-dependent manner.

A

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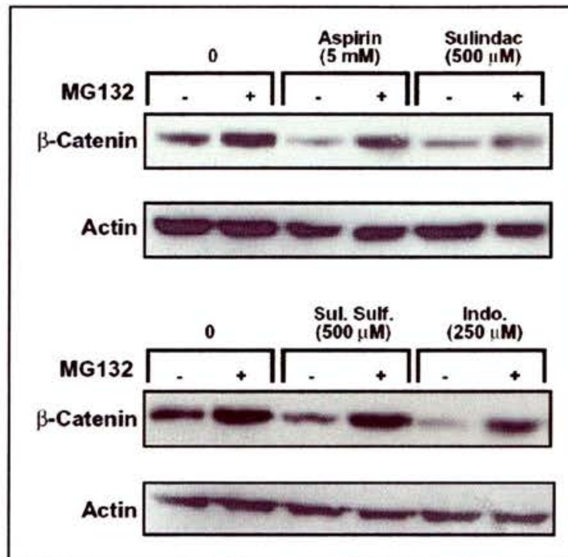



**Figure 5.1 – NSAIDs Induce Proteasome-dependent Reduction in Cytoplasmic β-Catenin.** **(A)** In three independent experiments, SW480 cells were treated for 16 hrs with Aspirin (0, 3 mM, 5 mM), Sulindac (0, 300 μM, 500 μM), Sulindac Sulfone (0, 300 μM, 500 μM), or Indomethacin (0, 125 μM, 250 μM) then cytoplasmic extracts (15 μg) were resolved by SDS PAGE on 8.5% polyacrylamide gels and anti-β-catenin Western blot carried out. Cu/Zn SOD was used as a loading control. Representative blots are shown. **(B)** In three independent experiments, SW480 cells were pre-treated with MG132 (25 μM) for 1 hr prior to overnight (16 hrs) treatment with Aspirin (0, 5 mM), Sulindac (500 μM), Sulindac Sulfone (Sul. Sulf.) (0, 500 μM), or Indomethacin (Indo.) (250 μM). Cytoplasmic extracts (15 μg) were resolved by SDS PAGE on 8.5% polyacrylamide gels and anti-β-catenin Western blot carried out. Actin was used as a loading control. Representative blots are shown.

It is well established that β-catenin is regulated and destabilised by a multiprotein complex comprising APC, GSK-3β, actin/conductin and CKI (Polakis, 2000). This multiprotein complex functions to phosphorylate β-catenin, targeting it for degradation via the ubiquitin pathway. Conversely, stabilised, hypophosphorylated β-catenin is free to translocate from the cytoplasm to the nucleus where it binds TCF/LEF and activates transcription of target genes.

To distinguish whether the reduction in cytoplasmic β-catenin levels in response to NSAID treatment was due to degradation or nuclear translocation of β-catenin, cells were treated with NSAIDs in the presence of the proteasome inhibitor, MG132. Figure 5.1B demonstrates that MG132 blocks the reduction in cytoplasmic β-catenin levels induced by aspirin, sulindac, sulindac sulfone and indomethacin, suggesting that these agents induce phosphorylation and degradation of β-catenin and that this likely involves ubiquitin ligation and targeting for proteasomal degradation.

### 5.2.2 β-catenin Localises to the Nucleolus After NSAID Treatment

To begin to determine the association between β-catenin and nuclear function of NF-κB, it was important to examine the subcellular localisation of β-catenin in response to NSAIDs. Since a decrease in cytoplasmic β-catenin was observed in response to NSAIDs, it was postulated that there would be little nuclear β-catenin after NSAID treatment. Using immunocytochemistry, it was found that β-catenin is predominantly cytoplasmic, concentrated at the cell membrane in untreated control

cells (Figure 5.2). However, treatment with aspirin, sulindac, sulindac sulfone and indomethacin caused β-catenin to accumulate in the nucleoplasm. Furthermore, β-catenin localised to distinct nuclear bodies, a feature that was particularly apparent for the higher concentrations of NSAIDs used, for example sulindac sulfone (500 μM) and indomethacin (250 μM). These nuclear bodies co-localised with areas devoid of DAPI staining. Moreover, for most treatments, these areas co-localised with the nucleolar protein, fibrillarin, indicating that β-catenin was actually accumulating in the nucleolus after NSAID treatment. However, it is noteworthy that at high concentrations of sulindac (500 μM) and aspirin (5 mM), fibrillarin appears to form a 'cap' around β-catenin. This could be due to disruption of the nucleoli with these agents, leading to the concentration of fibrillarin around the edge of the nucleolus. Similar to the observations for RelA, it is also noteworthy that at low concentration of NSAIDs, nucleolar β-catenin was more marked for sulindac sulfone (300 μM) and indomethacin (125 μM) compared with sulindac (300 μM) and aspirin (3 mM).

### 5.2.3 NSAIDs Induce Repression of TCF Transcriptional Activity

Having observed that β-catenin accumulated in the nucleolus in response to treatment with the NSAIDs, the next question to be addressed was whether this compartmentalization regulates nuclear TCF transcriptional activity. To do this, SW480 colon cancer cells were transiently transfected with a synthetic TCF reporter

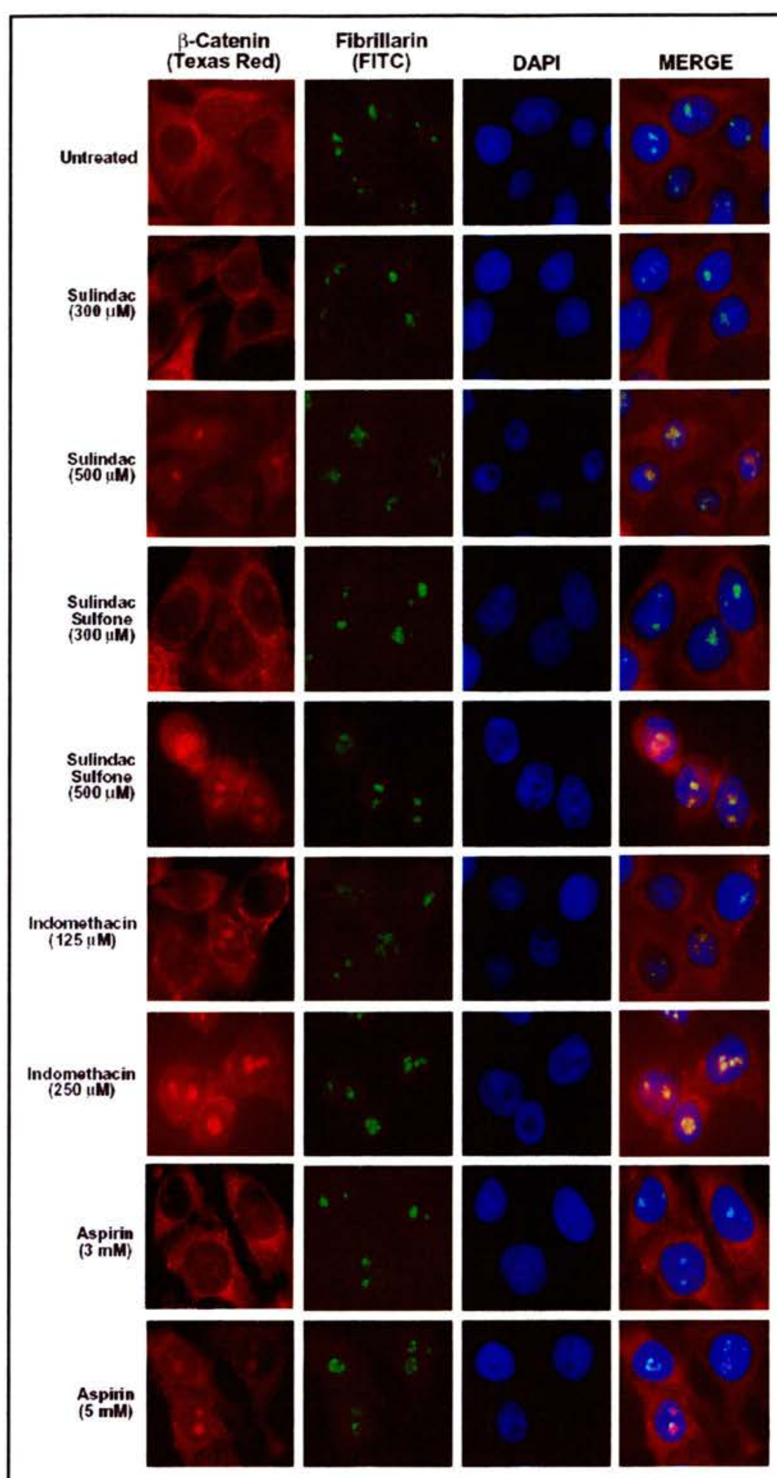



**Figure 5.2 – NSAIDs Induce Nucleolar Translocation of β-catenin.** SW480 cells were treated, in three independent experiments, for 16 hrs with Sulindac (300 μM, 500 μM), Sulindac Sulfone (300 μM, 500 μM), Indomethacin (125 μM, 250 μM), Aspirin (3 mM, 5 mM) or untreated (control). Representative micrographs (63 x) illustrating localisation of β-catenin and the nucleolar protein, fibrillarin, are shown.

plasmid, pTOPFlash (which consists of three TCF binding sites upstream of a minimal TK promoter and the Luciferase open reading frame) or pFOPFlash (which is identical to pTOPflash except that the TCF binding sites are mutated and inactive), along with the pCMVβ control plasmid, then treated with sulindac, sulindac sulfone, indomethecin and aspirin as indicated.

The data from these experiments are presented in Figure 5.3 and shows that the levels of relative β-catenin/TCF-driven luciferase activity from the pTOPFlash construct were lower following exposure to each of the NSAIDs compared to untreated controls. The decrease was concentration-dependent and ranged from 1.4 fold [sulindac sulfone (300 μM)] to 2.8 fold [Sulindac (500 μM)]. However, the NSAIDs had a similar effect on the transcription from the pFOPFlash control plasmid that has mutated TCF binding sites, although basal transcriptional levels were much lower, demonstrating that the observed decrease in transcriptional activity is not specific to TCF in response to these agents.

During the course of this PhD, several published reports demonstrated a reduction in levels of β-catenin/TCF-driven luciferase activity using the pTOPFlash construct in response to NSAIDs (Dihlmann *et al.*, 2001 and 2003; Gardner *et al.*, 2004; Boon *et al.*, 2004), but no change in the levels of pFOPFlash activity was observed in those studies. In the experiments outlined above, the observation that the pFOPFlash construct caused repression in my hands meant that I could not generate robust β-catenin/TCF-driven transcription data for the panel of NSAIDs. It should be noted that this repression was seen in each of the experiments I carried out. Given the published results in the literature, the reasons for this are not clear, despite best

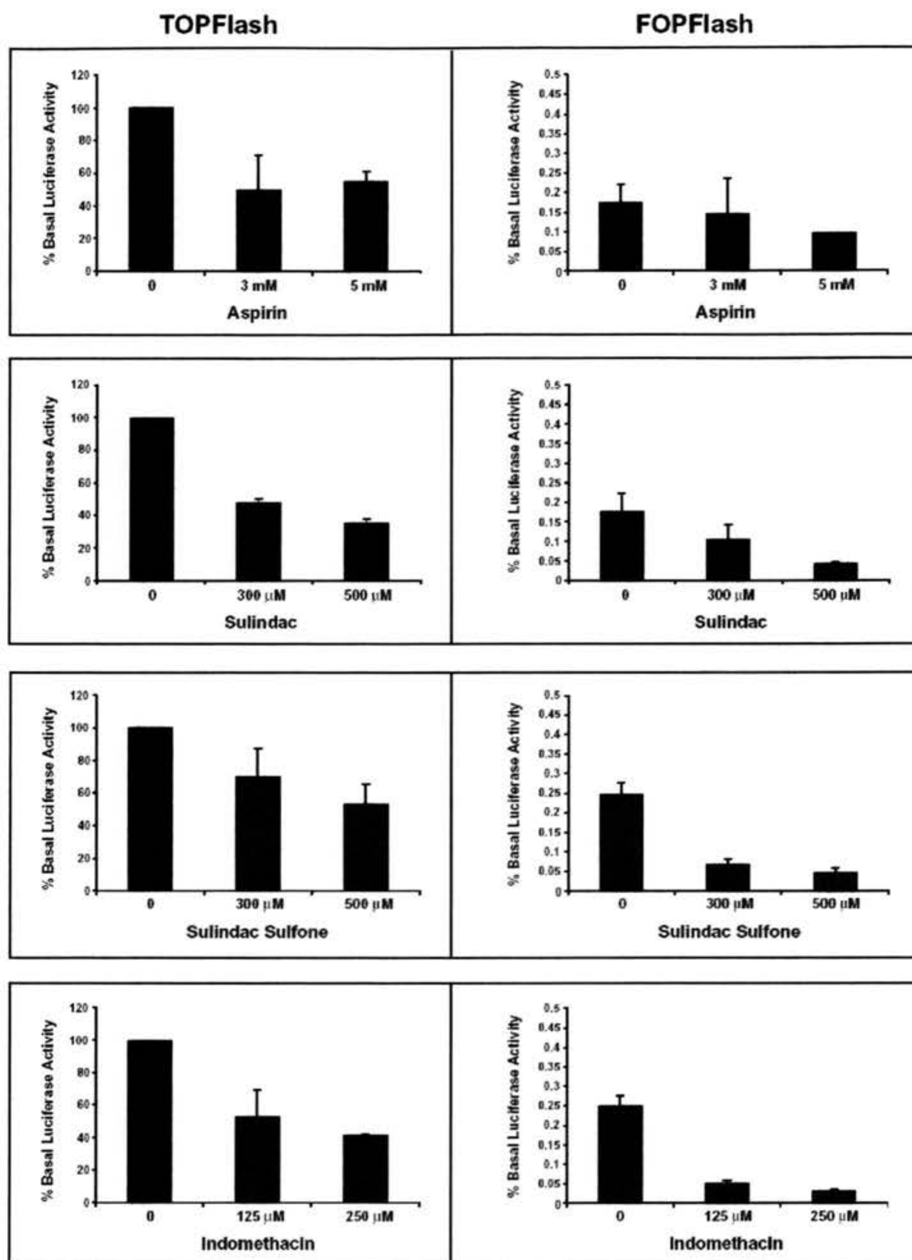



**Figure 5.3 – NSAIDs Induce Repression of TOPFlash and FOPFlash (β-catenin/TCF-driven) Luciferase Activity.** SW480 cells were transiently transfected with pTOPFlash or pFOPFlash and pCMVβ, then subsequently treated with Aspirin (0, 3 mM, 5 mM), Sulindac (0, 300 µM, 500 µM) Sulindac Sulfone (0, 300 µM, 500 µM) or Indomethacin (0, 125 µM, 250 µM). Luciferase assays were carried out to measure β-catenin/TCF transcriptional activity and β-galactosidase assays were used to normalise these results for transfection efficiency. % transcriptional activity was calculated relative to non-treated controls. FOPFlash activity is expressed relative to TOPFlash activity. The results presented are the mean of two independent experiments (+/- standard deviation).

efforts to resolve the problem in the time available. Hence, further work outwith the scope of this thesis is required to ascertain the effects of the panel of NSAIDs used in this study on β-catenin/TCF-driven transcription.

### 5.2.4 RelA and β-catenin do not Co-immunoprecipitate After Aspirin or Sulindac Treatment

Preliminary studies in the host laboratory suggested that β-catenin and RelA interact in the nucleus, but not in the cytoplasm, in response to aspirin (Stark *et al.*, 2003). Therefore, to further examine the association between β-catenin and regulation of the nuclear distribution of NF-κB, it was next important to confirm this interaction. SW480 colorectal cancer cells were treated overnight (16 hrs) with aspirin or sulindac and then immunoprecipitation for RelA or β-catenin, followed by Western blot analysis with an anti-RelA or anti-β-catenin antibody, was carried out using a standard method (see Chapter 2). The results shown in Figure 5.4 indicate that an interaction between β-catenin and RelA could not be detected after sulindac or aspirin treatment. Controls, however, demonstrated that RelA and β-catenin were specifically immunoprecipitated from the extract in an antibody-dependent manner. The discrepancy between these data and preliminary studies may be due to the conditions under which the immunoprecipitations were carried out. Whereas nuclear extracts were used in the preliminary studies, whole cell extracts were used in this study. Furthermore, the immunoprecipitation and cell lysis buffers used in this study

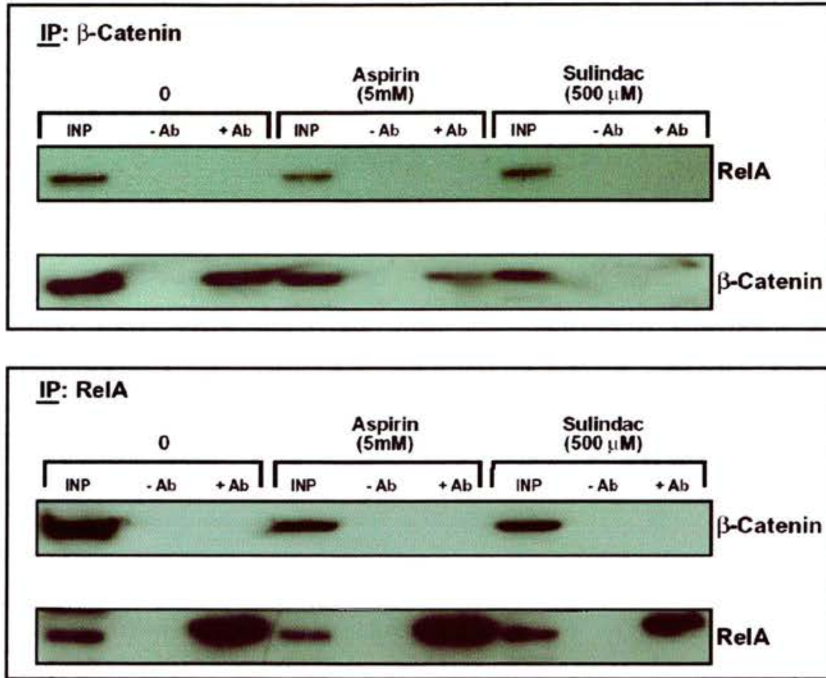



**Figure 5.4 – RelA and β-catenin do not Co-Immunoprecipitate After NSAID Treatment.**

In three independent experiments, SW480 cells were treated for 16 hr with Aspirin (0, 5 mM) or Sulindac (500 μM), then whole cell extracts (500 μg) immunoprecipitated (IP) using either anti-RelA (rabbit) antibody or anti-β-catenin (goat) antibody (+ Ab), or no antibody (- Ab) as a control. Inputs (INP) (30 μg) and immunoprecipitates were then resolved by SDS PAGE on 8.5% polyacrylamide gels and Western blot analysis, using anti-β-catenin (mouse) or anti-RelA (mouse) monoclonal antibodies, was subsequently carried out. Blots were then stripped and re-probed for β-catenin or RelA. Representative blots are shown.

had different components and salt concentrations compared to the buffers used in the preliminary studies, which could affect complex stability. Nevertheless, the data presented in this chapter indicate that RelA and β-catenin do not interact after aspirin or sulindac treatment. This result was disappointing given the exciting findings outlined below.

### 5.2.5 RelA and β-catenin Co-localise in Nucleolus after NSAID Treatment

Results from Chapter 3 and section 5.2.2 of this chapter strongly suggest that RelA and β-catenin co-localise in the nucleolus in response to NSAIDs. Therefore, in order to support this hypothesis, the next area of research was to determine the effects of NSAIDs on cellular localisation of β-catenin with respect to RelA. SW480 cells were treated overnight (16 hrs) with sulindac, sulindac sulfone, indomethacin or aspirin prior to harvesting and then immunocytochemistry employed to detect RelA and β-catenin. It can clearly be seen from the results in Figure 5.5 that β-catenin and RelA co-localise within distinct nuclear compartments, which have previously been shown to be the nucleolus, after NSAID treatment. This is particularly apparent for the higher concentrations of sulindac (500 μM), sulindac sulfone (500 μM), indomethacin (250 μM) and aspirin (5 mM). This data suggests that β-catenin and RelA could interact in the nucleolus after NSAID treatment. However, whether they translocate to the nucleolus independently or whether there is a direct interaction is still unclear.

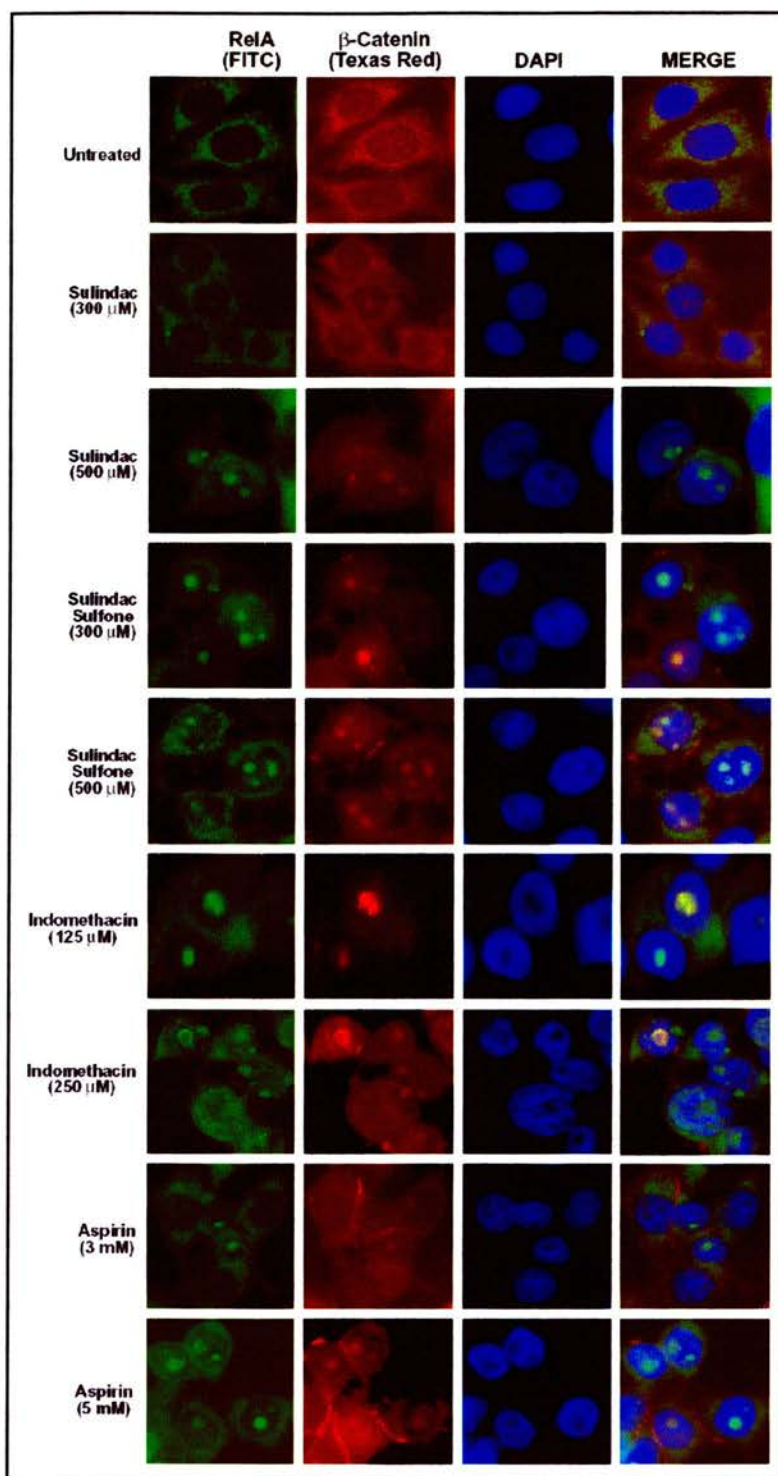



**Figure 5.5 – RelA and β-catenin Co-localise in the Nucleolus after NSAID Treatment.** In three independent experiments, SW480 cells were treated for 16 hrs with Sulindac (300 μM, 500 μM), Sulindac Sulfone (300 μM, 500 μM), Indomethacin (125 μM, 250 μM), Aspirin (3 mM, 5 mM) or untreated (control). Representative micrographs (63 x) illustrating localisation of RelA and β-catenin are shown.

### 5.2.6 RelA(Δ27-30) Blocks Nucleolar Localisation of β-catenin After NSAID Treatment

Previous work from the host laboratory demonstrated that in cultures expressing RelA deleted for the nucleolar localisation signal [RelA(Δ27-30)], there was no nucleolar sequestration of either mutant or endogenous protein in transfected cells, indicating that mutants which are unable to localise to the nucleolus act in a dominant negative fashion to block the nucleolar translocation of endogenous RelA (Stark and Dunlop, 2005). Furthermore, it was discovered in Chapter 3 that cells transfected with RelA(Δ27-30) did not exhibit nucleolar localisation of mutant protein in response to sulindac, sulindac sulfone or indomethacin. The RelA(Δ27-30) dominant negative mutant was therefore used to investigate the co-dependency of nucleolar sequestration of RelA and β-catenin in response to NSAIDs.

SW480 cells were transiently transfected with GFP-RelA(WT) or GFP-RelA(Δ27-30) prior to overnight treatment with aspirin (16 hrs) and then immunocytochemistry subsequently performed to detect β-catenin. From the results in Figure 5.6A, β-catenin and GFP-RelA(WT) were predominantly cytoplasmic in untreated control cells but co-localised in the nucleolus in response to aspirin treatment, as previously demonstrated. It is noteworthy that there was a degree of nuclear GFP-RelA(WT) (18-19%) both in untreated and aspirin treated cells (Figure 5.6B). More importantly, there was an increase in nucleolar GFP-RelA(WT) from ~1% in untreated cells to 55% after aspirin treatment and a corresponding increase in nucleolar β-catenin in transfected cells from ~1% to 53% after aspirin treatment. In contrast to GFP-RelA(WT), GFP-RelA(Δ27-30) was predominantly cytoplasmic or

nuclear and β-catenin mainly cytoplasmic both in untreated control cells and after aspirin treatment (Figure 5.6A). There was an increase in nuclear GFP-RelA(Δ27-30) levels after aspirin treatment from 43% to 61% (Figure 5.6B). However, only a very small increase in nucleolar levels of GFP-tagged RelA and β-catenin in transfected cells was observed, with maximum levels of both proteins in the nucleolus reaching just ~8% after aspirin treatment. Furthermore, the effect of the RelA(Δ27-30) mutant was global as untransfected cells did not display nucleolar β-catenin in response to aspirin treatment. This data strongly suggests that RelA interacts with β-catenin and that nucleolar sequestration of RelA is required for localisation of β-catenin to the nucleolus.

## 5.3 Discussion

The strong similarities between the NF-κB and Wnt/β-catenin pathways, combined with the findings of several recent studies which have shown that β-catenin can regulate nuclear NF-κB and vice versa (Deng *et al.*, 2002; Masui *et al.*, 2002; Deng *et al.*, 2004), implicate β-catenin as a good candidate for regulating nuclear NF-κB in response to NSAIDs and while accepting that it is far from conclusive, the data presented in this chapter supports this notion. The evidence to substantiate this is based on the following observations: **1**. NSAIDs mediate a proteasome-dependent reduction in cytoplasmic β-catenin levels (Figure 5.1) and induce nucleolar localisation of β-catenin (Figure 5.2). **2**. RelA and β-catenin co-

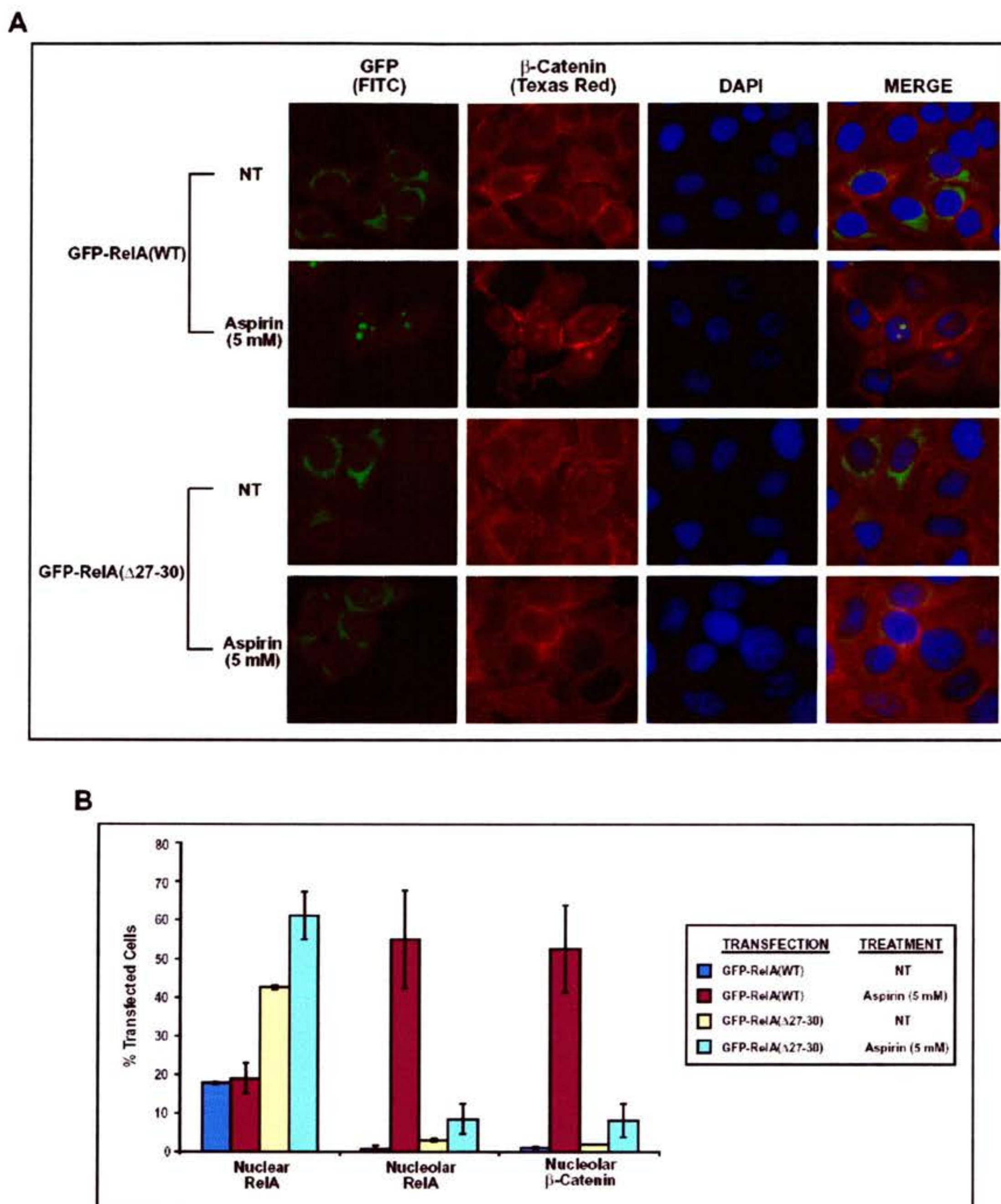



**Figure 5.6 – RelA(Δ27-30) Blocks Nucleolar Sequestration of β-catenin.** **(A)** SW480 cells were transiently transfected, in two independent experiments, with either GFP-RelA(WT) or GFP-RelA(Δ27-30) then treated for 16 hrs with Aspirin (5 mM) or non-treated (NT) (control). Representative micrographs (63 x) illustrating localisation of GFP and β-catenin are shown. **(B)** The percentage of transfected cells expressing nuclear RelA, nucleolar RelA and nucleolar β-catenin was determined by fluorescence microscopy in a minimum of 200 cells from multiple fields of view. The results presented are the mean of two independent experiments (+/- standard deviation).

localise in the nucleolus (Figure 5.5). **3**. RelA(Δ27-30) blocks aspirin-induced nucleolar sequestration of β-catenin (Figure 5.6). It should be noted, however, that data regarding NSAID effects on β-catenin/TCF-driven transcriptional activity was inconclusive (Figure 5.3) and that a direct interaction between RelA and β-catenin was not observed after aspirin or sulindac treatment (Figure 5.4).

The discovery that NSAIDs caused a proteasome-dependent reduction in cytoplasmic β-catenin levels (Figure 5.1) suggested that β-catenin was phosphorylated, and subsequently degraded via the ubiquitin-proteasome pathway. β-catenin is known to be phosphorylated at key serine and threonine residues by a multiprotein complex comprising axin or conductin, GSK-3β, APC and CKI (Sieber *et al.*, 2000). Phosphorylation of β-catenin by this complex targets it for ubiquitination by the E3 ubiquitin ligase, β-TrCP, and subsequent degradation by the proteasome pathway (Lustig and Behrens, 2003). Therefore, the effects of NSAIDs may be to induce phosphorylation of β-catenin by activating upstream kinases, and these could represent a point of cross-regulation with the NF-κB pathway. GSK-3β and IKK are of particular interest as these kinases have been shown to phosphorylate both β-catenin (Lamberti *et al.*, 2001) and IκB (Hoeflich *et al.*, 2000). GSK-3β is a strong candidate because inhibition of GSK-3β has been shown to suppress activation of NF-κB (Deng *et al.*, 2004). Having established in the previous two chapters that non-aspirin NSAIDs activate the NF-κB pathway by a mechanism that is independent of IκB degradation, it seems unlikely that IKK plays a significant role in activation and cross-regulation of the NF-κB and β-catenin pathways in response to NSAID treatment. On the other hand, GSK-3β remains an interesting candidate target for NSAIDs that may be worthy of further research beyond the scope of this

thesis. Besides activation of upstream kinases, another possibility is that NSAIDs induce ubiquitination of β-catenin via activation of β-TrCP, which could also mediate cross-talk with NF-κB as it is a common E3 ubiquitin ligase with IκB (Amit and Ben Neriah, 2003).

The data presented in this chapter regarding NSAID effects on cytoplasmic β-catenin are in keeping with results from previous studies. One paper (Gardner *et al.*, 2004) reported a time and dose-dependent reduction of β-catenin in response to a panel of NSAIDs; another report established a dose-dependent down-regulation of β-catenin protein in response to indomethacin (Veeramachaneni *et al.*, 2003); and two studies established that sulindac sulfone induced a reduction of β-catenin in a time- and dose-dependent manner (Thompson *et al.*, 2000; Li *et al.*, 2002). Furthermore, it has been determined that sulindac metabolites induce caspase- and proteasome-dependent degradation of β-catenin (Rice *et al.*, 2003). However, in contrast to these studies, two papers documented that there was no change in β-catenin protein levels in response to aspirin or indomethacin (Dihlmann *et al.*, 2001 and 2003). It is important to note that a key difference between the experiments described here and the published studies described above is that cytoplasmic and not total protein was found to be reduced in a dose- and proteasome-dependent manner in response to aspirin, sulindac, sulindac sulfone and indomethacin. The favoured hypothesis is that cytoplasmic β-catenin is phosphorylated by an upstream kinase, such as GSK-3β, and subsequently degraded by the ubiquitin-proteasome pathway in response to NSAIDs.

The novel observation that β-catenin accumulates in the nucleolus after treatment with aspirin, sulindac, sulindac sulfone and indomethacin (Figure 5.2) was

surprising given the previous result that cytoplasmic β-catenin is degraded in response to these agents. One possible explanation for these findings is that there are two pools of β-catenin: a cytoplasmic pool and a nuclear pool. Non-treated cells do exhibit some general nuclear staining of β-catenin and colorectal cancer cells are known to have high nuclear β-catenin activity caused by mutation of *APC* (Korinek *et al.*, 1997). One could further speculate, therefore, that it is the nuclear pool of β-catenin which accumulates within the nucleolus upon NSAID treatment.

Previous studies have investigated the effects of NSAIDs on localisation of β-catenin, but have provided inconsistent results. Two studies (Thompson *et al.*, 2000; Li *et al.* 2002) demonstrated a general decrease in cytoplasmic and nuclear β-catenin levels in response to treatment with sulindac sulfone, and another report found that after sulindac treatment, nuclear β-catenin staining is reduced in adenomas of FAP patients (Boon *et al.*, 2004). It has been established that sulindac sulfide inhibits nuclear β-catenin staining (Rice *et al.*, 2003) and that there is a decrease in cytoplasmic and nuclear β-catenin staining, but increased membranous staining, in response to indomethacin (Kapitanović *et al.*, 2006). Similarly, it was discovered that diclofenac, indomethacin and sulindac sulfide cause increased membranous β-catenin staining with decreased cytoplasmic and nuclear staining (Gardner *et al.*, 2004). In contrast to these studies, one report documented no change in distribution or intensity of β-catenin staining in response to aspirin or indomethacin (Dihlmann *et al.*, 2001). Rofecoxib has also been shown to have a minimal reduction in nuclear β-catenin signal (Gardner *et al.*, 2004). The finding here in this study that β-catenin localises to the nucleolus in response to NSAIDs supports the notion that β-catenin plays a role in the nuclear distribution of RelA.

Having observed that β-catenin accumulates in the nucleolus after NSAID treatment, it was postulated that this subcellular localisation could affect β-catenin/TCF-driven transcriptional activity, in an analagous manner to nucleolar localisation of RelA and repression of NF-κB-driven transcription. However, no robust data regarding NSAID effects on β-catenin/TCF-driven transcription was generated using the synthetic TCF reporter plasmids, pTOPFlash and pFOPFlash, due to the fact that repression of luciferase activity was observed with both constructs in response to the panel of NSAIDs (see 5.2.3). Given the technical problems encountered with pFOPFlash, it would be important in the future to make use of a different approach to control for specificity of effect on TCF-driven transcriptional activity in response to NSAIDs. Moreover, it might be informative to investigate expression of a few downstream genes.

Although problems were encountered with pFOPFlash in my hands, several published studies have documented repression of β-catenin/TCF-driven transcriptional activity in response to NSAIDs using the pTOPFlash/pFOPFlash constructs. Aspirin and indomethacin have been shown to down-regulate β-catenin/TCF signaling in a dose-dependent manner (Dihlmann *et al.*, 2001 and 2003); sulindac sulfide was demonstrated to inhibit TCF transcriptional activation (Boon *et al.*, 2004); doxycycline and indomethacin have been reported to synergistically down-regulate β-catenin signaling (Veeramachaneni *et al.*, 2003); and diclofenac and sulindac sulfide, but not indomethacin, sulindac sulfone or rofecoxib, were established to mediate a reduction in catenin-related transcription (Gardner *et al.*, 2004). Previous results published from the host laboratory led to the hypothesis that the decrease in NF-κB-driven transcription observed in response to aspirin was

caused by basal NF-κB complexes being disturbed from DNA and subsequently translocating to the nucleolus (Stark and Dunlop, 2005). In contrast, the data presented here suggest that it is the β-catenin fraction not bound to TCF at promoter sites that translocates to the nucleolus. One possible way to investigate this hypothesis would be to carry out chromatin immunoprecipitation (CHIP) assays, examining promoters of known TCF target genes in response to NSAIDs.

This study is the first to demonstrate nucleolar sequestration of β-catenin, although the mechanism and consequence of this event is unclear. Key questions relate to the possible function of β-catenin in the nucleolus and secondly the mechanism by which it gets there. As was discussed previously for RelA, it is increasingly recognised that localisation of transcription factors and/or their co-factors to the nucleolus is involved in regulation of the cell cycle and apoptosis. Nucleolar accumulation can be mediated by the presence of a specific nucleolar targeting sequence or molecular interaction with a nucleolar interaction partner (Scheer and Hock, 1999; Olson, 2004). There is no known nucleolar targeting sequence for β-catenin but it is of interest that there is a PSQR (proline-serine-glutamine-arginine) motif at position 371 in β-catenin, which is very similar to the PKQR (proline-lysine-glutamine-arginine) motif that was identified in RelA as the nucleolar localisation signal (Stark and Dunlop, 2005). Another possibility is that molecular interaction with another molecule is required for β-catenin to localise to the nucleolus. However, in contrast to RelA, no nucleolar proteins like ARF, B23 or NFBP have been shown to interact with β-catenin. Given that RelA localises to the nucleolus (Stark and Dunlop, 2005) and that β-catenin can directly interact with

RelA (Deng *et al.*, 2002), it was postulated that β-catenin is targeted to the nucleolus by RelA.

Preliminary studies in the host laboratory demonstrated that β-catenin can interact with RelA in the nucleus in response to aspirin treatment (Stark *et al.*, 2003). As an initial step to establish whether RelA targets β-catenin to the nucleolus, it was important to confirm these preliminary data. However, an interaction between RelA and β-catenin could not be detected after aspirin or sulindac treatment by immunoprecipitation (Figure 5.4). The next strategy was therefore to determine whether co-localisation occurred between RelA and β-catenin, a result that was implied from previous observations in this study. Immunocytochemistry demonstrated that RelA and β-catenin co-localised in the nucleolus in response to aspirin, sulindac, sulindac sulfone and indomethacin (Figure 5.5), suggesting that an interaction between the two proteins could occur there. It should be noted that there is a little discrepancy between these data and those presented in Figures 3.3 and 5.2 for 300 μM sulindac and 125 μM indomethacin – only nuclear RelA is shown in Figure 3.3 when cells were treated with 300 μM sulindac but nucleolar RelA is evident in Figure 5.5; nucleolar β-catenin is more prominent in cells shown in Figure 5.5 that were treated with 300 μM sulindac when compared to those illustrated in Figure 5.2; only one cell in four treated with 125 μM indomethacin has nucleolar β-catenin in Figure 5.2 but both cells treated with 125 μM indomethacin in Figure 5.5 display nucleolar β-catenin. In hindsight, it would therefore have been a good idea to quantitate the numbers of cells exhibiting nuclear and nucleolar RelA and/or β-catenin to allow the use of statistics in order to clearly demonstrate effects.

It is noteworthy that data presented in Figure 5.5 demonstrating co-localisation of RelA and β-catenin in the nucleolus could provide a possible explanation for the failure to detect an interaction between RelA and β-catenin in the co-immunoprecipitation experiment outlined in Figure 5.4. Neither nuclear nor nucleolar, but whole cell extracts were used in co-immunoprecipitation experiments and so it is conceivable that there was not sufficient nucleolar protein present in these extracts to detect an interaction between RelA and β-catenin if the nucleolus is where they interact. Furthermore, many unstable protein complexes, particularly nuclear protein complexes, can be affected by the salt concentration of the buffer used in the immunoprecipitation process. It is worth mentioning that the salt concentration of the whole cell extraction buffer employed in the co-immunoprecipitation experiment outlined in Figure 5.4 was very low (150 mM) and so so the use of a higher salt concentration, perhaps 350-400 mM, to increase the stringency of the buffer may also have aided detection of an interaction between RelA and β-catenin.

To begin to address the question of whether RelA and β-catenin localise to the nucleolus independently or are required to interact in order to be sequestered in the nucleolus, the RelA(Δ27-30) dominant negative mutant was used. Blocking aspirin-induced nucleolar translocation of RelA using this construct blocked nucleolar translocation of β-catenin (Figure 5.5), strongly indicating that interaction of RelA and β-catenin is required for nucleolar localisation of these proteins. However, it would be important in the future to establish a control protein whose nucleolar localisation is unaffected by the RelA(Δ27-30) construct in order to demonstrate the specificity of its effect on β-catenin and that it does not cause general disruption of nucleolar proteins. Moreover, although providing evidence to

the contrary, the possibility that RelA and β-catenin localise to the nucleolus independently cannot be completely excluded from the results of that experiment alone as it is possible that by blocking nucleolar translocation, the RelA construct could cause a common carrier to be sequestered in the nucleus, thus preventing β-catenin from going to the nucleolus. However, given that β-catenin has not previously been reported to interact with any other well-established nucleolar proteins, it seems most likely that RelA directly carries β-catenin to the nucleolus.

There has only been one study to date investigating the effects of NSAIDs on interaction of the NF-κB and β-catenin pathways. The NSAID diclofenac has been reported to inhibit Wnt/β-catenin signaling, without altering β-catenin protein levels, and reduce the expression of β-catenin/TCF-dependent genes in colon cancer cells (Cho *et al.*, 2005). In that study, diclofenac induced activation of the NF-κB pathway through degradation of IκBα, which increased free nuclear NF-κB. Ectopic expression of RelA suppressed β-catenin/TCF-mediated transcriptional activity, suggesting that diclofenac inhibits Wnt/β-catenin signaling via activation of the NF-κB pathway. However, in contrast to that published report, the data presented here demonstrates that non-aspirin NSAIDs activate NF-κB by a mechanism that is independent of IκBα degradation (Chapter 3) and that NSAIDs mediate a proteasome-dependent decrease in cytoplasmic β-catenin levels (section 5.2.1).

Several studies support the notion that interaction of NF-κB and β-catenin can regulate nuclear activity of RelA and/or β-catenin. It has previously been observed that β-catenin physically interacts with RelA and that this results in a reduction in NF-κB DNA binding, transactivation activity and target gene expression (Deng *et al.*, 2002). A more recent study by the same group demonstrated that

inhibition of GSK-3β suppressed NF-κB activity in response to TNF-α (Deng *et al.*, 2004). It has also been shown that RelA specifically represses β-catenin/TCF-dependent transcription, an effect that is independent of the DNA binding ability of β-catenin/TCF complex and the trans-acting transcriptional ability of RelA (Masui *et al.*, 2002). It could therefore be envisaged that interaction of RelA and β-catenin may be, at least in part, responsible for apoptosis induced by NSAIDs via alteration of NF-κB and/or β-catenin/TCF transcriptional activity and hence expression of target genes.

Collectively, the data presented in this chapter provides evidence to support the notion that the β-catenin pathway is influenced by aspirin, sulindac, sulindac sulfone and indomethacin. Specifically, the favoured hypothesis is that these agents induce degradation of cytoplasmic β-catenin and nucleolar sequestration of nuclear β-catenin. Moreover, the nucleolus may represent a key site for interaction between the NF-κB and β-catenin pathways. Such cross-talk might serve as a very important regulatory mechanism in the apoptotic response to NSAIDs and hence could be considered in the design of novel anti-cancer agents.

# Chapter 6 – NSAID Effects on Post-translational Modifications of RelA and Relevance to Nucleolar Localisation

## 6.1 Introduction

It is now well established that the nuclear activity of RelA is subject to regulation by post-translational modifications (reviewed in Chapter 1). In particular, acetylation of RelA itself, or histones surrounding RelA, has been strongly implicated in regulation of NF-κB activity [reviewed in (Quivy and Van Lint, 2004; Schmitz *et al.*, 2004; Perkins and Gilmore, 2006)]. Direct acetylation of NF-κB subunits RelA and p50 has been shown to regulate NF-κB transcriptional activation, NF-κB DNA-binding affinity and IκBα assembly [reviewed in (Chen and Greene, 2003)]. Deacetylase inhibitors such as TSA or sodium butyrate can enhance NF-κB-dependent gene expression in the presence of TNFα (Ashburner *et al.*, 2001; Chen *et al.*, 2001; Inan *et al.*, 2000a). Furthermore, acetylation of histones regulates accessibility of NF-κB to target genes (Sheppard *et al.*, 1999) and acetyltransferases and deacetylases interact with several proteins involved in the NF-κB pathway, including IκBα and IKK (Viatour *et al.*, 2003; Yamamoto *et al.*, 2003).

Ubiquitination as a pathway for targeting RelA for destruction is also a potential regulatory mechanism and recent evidence has shown that RelA is indeed subject to regulation by ubiquitination. One study (Ryo *et al.*, 2003) demonstrated that NF-κB signaling is subject to regulation by Pin-1-dependent prolyl isomerisation

and ubiquitin-mediated proteolysis of RelA, which is facilitated by the E3 ubiquitin ligase, SOCS-1. Additionally, it has been documented that degradation of promoter-bound RelA is essential for the termination of the NF-κB pathway in response to TNFα (Saccani *et al.*, 2004).

The discovery that RelA is subject to regulation by ubiquitination is of particular interest because the nucleolus has been implicated as a potential site for proteasome mediated degradation of several proteins, including B23 (Itahana *et al.*, 2003), c-Myc (Arabi *et al.*, 2003; Welcker *et al.*, 2004) and Survivin-deltaEx3 (Song and Wu, 2005). Furthermore, accumulation of proteasomes at the nucleoli of cells containing elevated c-Myc protein levels has been reported (Arabi *et al.*, 2003). Proteins associated with the nuclear structure promyelotic leukaemia (PML) oncogenic domains (PODs or PML bodies) have been shown to move to the nucleolus upon proteasome inhibition (Mattsson *et al.*, 2001). Moreover, several studies have shown that PODs are involved in the proteasomal degradation of ubiquitinated proteins (Everett, 2000; Everett *et al.*, 1997; Wójcik and DeMartino, 2003; Smith *et al.*, 2004; Fabunmi *et al.*, 2001; Lallemand-Breitenbach *et al.*, 2001).

Given that RelA has been shown to be subject to regulation by the post-translational modifications discussed above, and that the nucleolus has been implicated as a potential site for ubiquitin mediated proteolysis of specific proteins, the aim of this chapter was to determine the role of ubiquitination and acetylation in the sequestration and function of RelA in the nucleolus after NSAID treatment.

## 6.2 Results

### 6.2.1 MG132 Induces Nucleolar Localisation of RelA

Since RelA has been shown to be subject to regulation by ubiquitin mediated proteolysis (Ryo *et al.*, 2003; Saccani *et al.*, 2004) and the nucleolus has been identified as a site for proteasome-mediated degradation (Itahana *et al.*, 2003; Arabi *et al.*, 2003; Welcker *et al.*, 2004; Song and Wu, 2005), the proteasome inhibitor MG132 was used to investigate the role of ubiquitination of RelA in nucleolar sequestration of RelA. Initially, immunocytochemistry was used to examine the subcellular localisation of RelA in SW480 colon cancer cells treated for 16 hrs with MG132. The results presented in Figure 6.1 show that RelA is predominantly cytoplasmic in untreated control cells. In response to MG132, RelA accumulated in the nucleoplasm and localised to distinct nuclear bodies. These nuclear bodies were located in areas devoid of DAPI staining. Furthermore, they co-localised with areas of antibody staining for the nucleolar protein, C23, thus confirming that RelA accumulates in the nucleolus after treatment with MG132.

### 6.2.2 MG132 Induces Repression of NF-κB Transcriptional Activity

In light of the exciting observation above, the next focus of investigation was to determine whether, as is the case for the NSAIDs aspirin, sulindac, sulindac

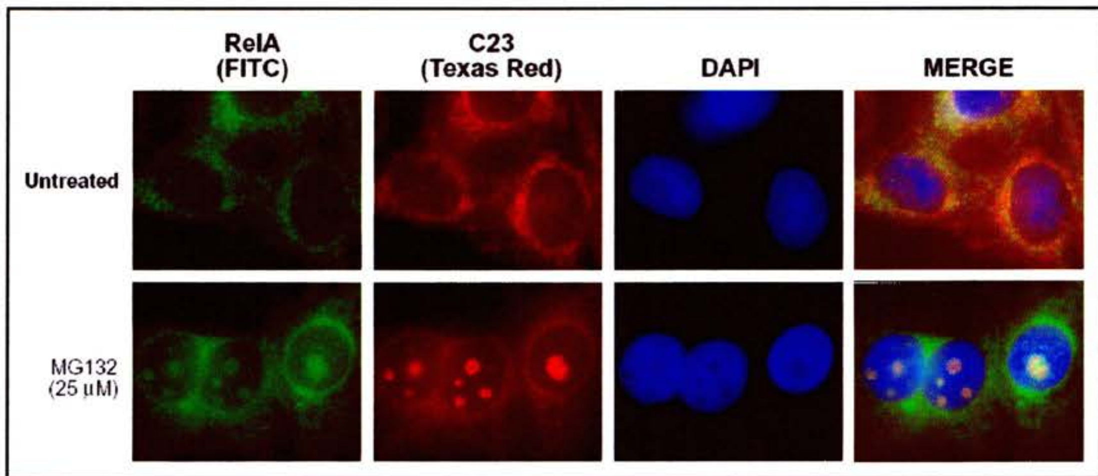



**Figure 6.1 – RelA Localises to the Nucleolus After MG132 Treatment.** In three independent experiments, SW480 cells were treated for 16 hrs with MG132 (25 μM) or untreated (control). Representative micrographs (63 x) illustrating localisation of RelA and the nucleolar protein, C23, are shown.

sulfone and indomethacin, compartmentalization of RelA in the nucleolus in response to MG132 is associated with changes in NF-κB driven transcriptional activity. To that end, SW480 colon cancer cells were transiently transfected with the 3x κB ConA-Luc NF-κB dependent luciferase reporter plasmid, along with the pCMVβ control plasmid, then treated with MG132 for 16 hrs. Strikingly, Figure 6.2A demonstrates that MG132, like the NSAIDs, causes a decrease (3.3 fold) in NF-κB-driven luciferase activity. Furthermore, MG132 did not cause a decrease in the relative luciferase activity when cells were transiently transfected with the ConA-Luc control plasmid that lacks κB sites, indicating the observed decrease in activity with the 3x κB ConA-Luc plasmid effect is specific (Figure 6.2B).

Collectively, the data presented so far indicates that MG132 mediates nucleolar translocation of RelA and repression of NF-κB-driven transcriptional activity. These results are very similar to the data presented for NSAIDs. However, it should be noted that the mechanisms utilised by NSAIDs could be different to MG132 and this will be discussed later.

### 6.2.3 MG132 and Aspirin Mediate an Increase in Ubiquitinated RelA

To investigate the role of ubiquitination in nucleolar translocation of RelA, SW480 cells were treated for 16 hrs with MG132 or aspirin and immunoprecipitation carried out using an anti-RelA antibody. Western blot analysis of the

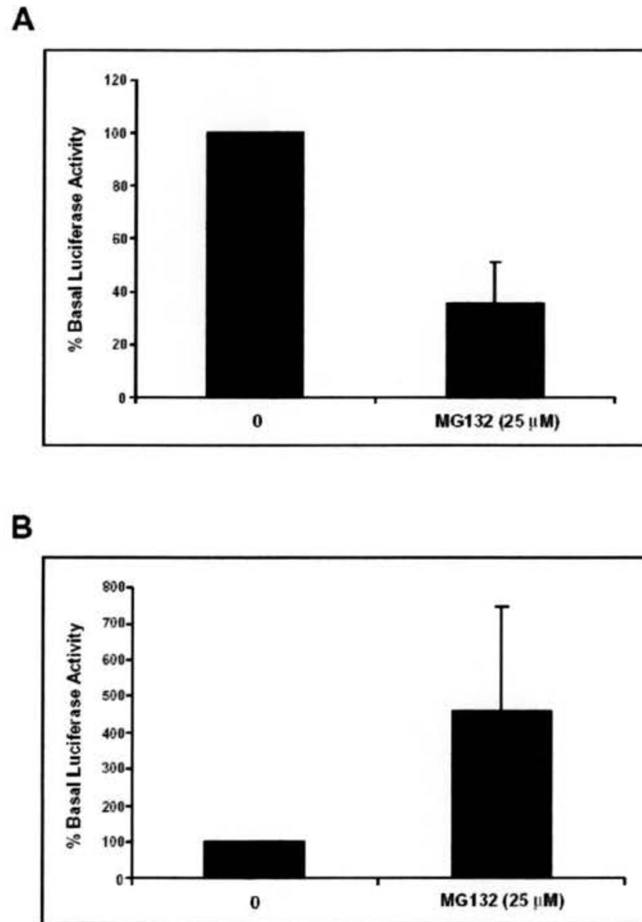



**Figure 6.2 – MG132 Induces Repression of NF-κB Transcriptional Activity.** SW480 cells were transiently transfected with (A) 3x κB ConA-Luc and pCMVβ or (B) ConA-Luc and pCMVβ, then treated with MG132 (0, 25 μM). Luciferase assays were carried out to measure NF-κB transcriptional activity and β-galactosidase assays were used to normalise these results for transfection efficiency. % transcriptional activity was calculated relative to non-treated controls. The results presented are the mean of three independent experiments (+/- standard deviation).

immunoprecipitated samples performed with an anti-ubiquitin antibody indicated that there is an increase in total ubiquitination after treatment with MG132 when compared to the untreated control (compare tracks 1 and 4 in Figure 6.3A, particularly the area highlighted by red arrows). This was expected as blocking proteasome activity leads to an accumulation of ubiquitin-conjugated proteins. Commensurate with a general increase in ubiquitinated proteins on treatment with MG132, there was also an increase in ubiquitin-conjugated RelA (compare tracks 3 and 6 in the region outlined for ubiquitin-conjugated RelA in Figure 6.3A). The observation that RelA is ubiquitinated following exposure to MG132 is in keeping with previously published work reporting that regulation of RelA can occur through the ubiquitination pathway (Ryo *et al.*, 2003; Saccani *et al.*, 2004). Similar to MG132, aspirin caused an increase in general protein ubiquitination (compare tracks 1 and 2 in Figure 6.3B, particularly the area highlighted by red arrows), but this was more subtle than the effect observed for MG132. Again, in keeping with a general increase in ubiquitinated proteins after aspirin treatment, there was also an increase in ubiquitin-conjugated RelA (compare tracks 5 and 6 in the region outlined for ubiquitin-conjugated RelA in Figure 6.3B). Although these data show that aspirin increases ubiquitin-conjugated RelA, the degree and specificity of this effect cannot be assessed from this experiment alone. Nonetheless, these results are intriguing and will be the subject of further work.

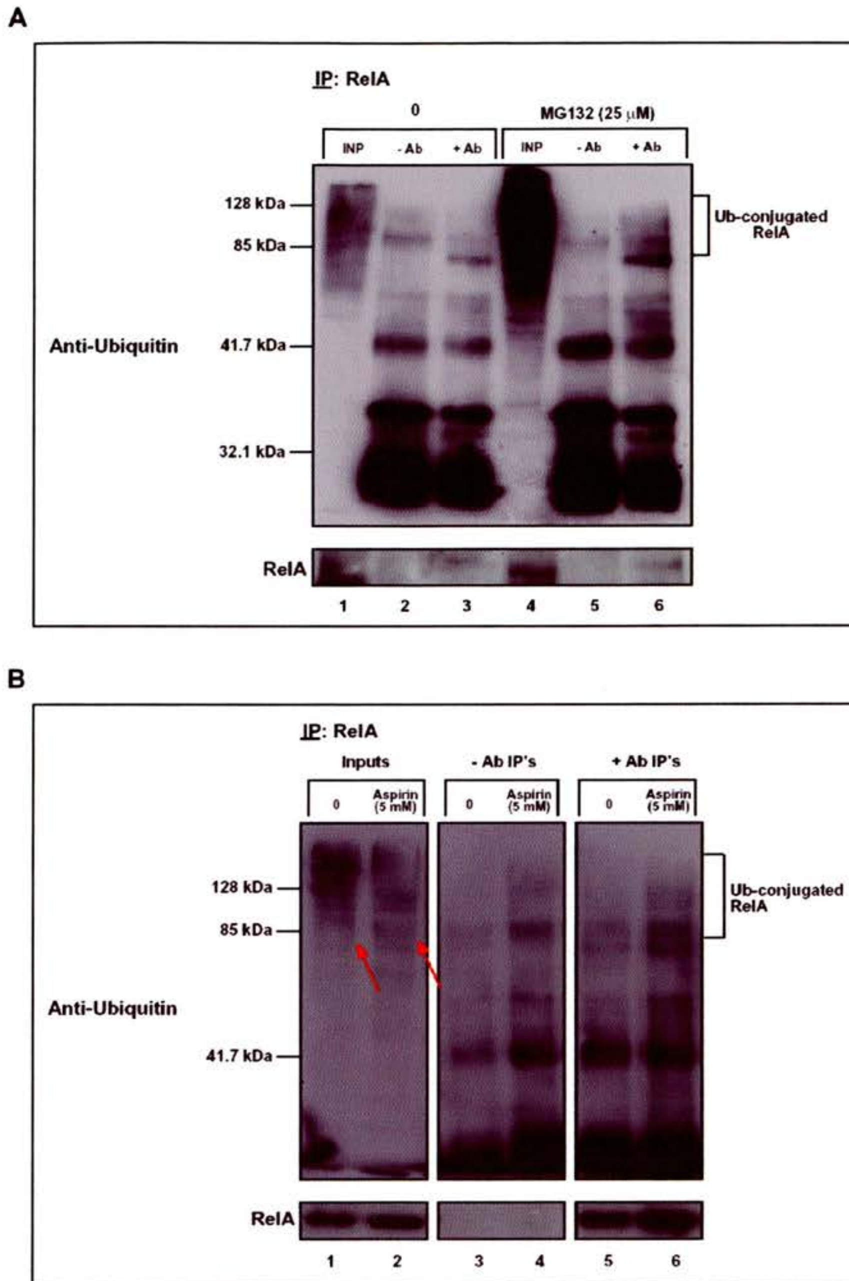



**Figure 6.3 – MG32 and Aspirin Induce an Increase in Ubiquitinated RelA.** SW480 cells were treated, in three independent experiments, for 16 hrs with **(A)** MG132 (0, 25 μM) or **(B)** Aspirin (0, 5 mM) then whole cell extracts (500 μg) immunoprecipitated (IP) using ant-RelA (mouse) antibody (+ Ab) or no antibody (- Ab) as a control. Inputs (INP) (30 μg) and immunoprecipitates were resolved by SDS PAGE on 8% polyacrylamide gels then Western blot analysis using anti-ubiquitin antibody subsequently carried out. Blots were stripped and re-probed with anti-RelA (rabbit) antibody. Representative blots are shown. Red arrows highlight areas of total protein ubiquitination described in the text. It should be emphasized that the gels presented in **(A)** and **(B)** were loaded differently. N.B. the same tracks are present, but just in a different order.

### 6.2.4 Total Proteasome Levels are Reduced by Aspirin and MG132

Given that both MG132 and aspirin increased the ubiquitin status of RelA, this suggested that aspirin behaves, to a certain extent, like the proteasome inhibitor to either alter proteasome levels and/or proteasome activity. Therefore, the effects of aspirin and MG132 on the total cellular levels of proteasomes was next investigated. To that end, Western blot analysis using protein extracts from SW480 cells treated with aspirin or MG132 for 16 hrs was employed. From the results, it was found that aspirin and MG132 both cause a significant reduction in total proteasome levels (Figure 6.4). This data indicates that aspirin behaves in a similar manner to the proteasome inhibitor to reduce proteasome levels and hence proteasome activity. While this is a very exciting result for aspirin, it should again be noted that the degree of specificity of the effect cannot be determined solely from this experiment but would warrant further work beyond the scope required for this thesis.

### 6.2.5 Proteasomes Localise to the Nucleolus in Response to Aspirin and MG132

As components of the ubiquitin system are not evenly distributed throughout the cell (Lenk and Sommer, 2000), the localisation of proteasomes in response to aspirin and MG132 was studied in order to further investigate the hypothesis that RelA is subject to ubiquitin-mediated degradation in the nucleolus in response to

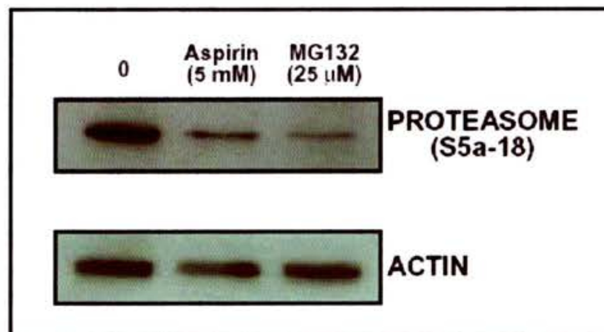
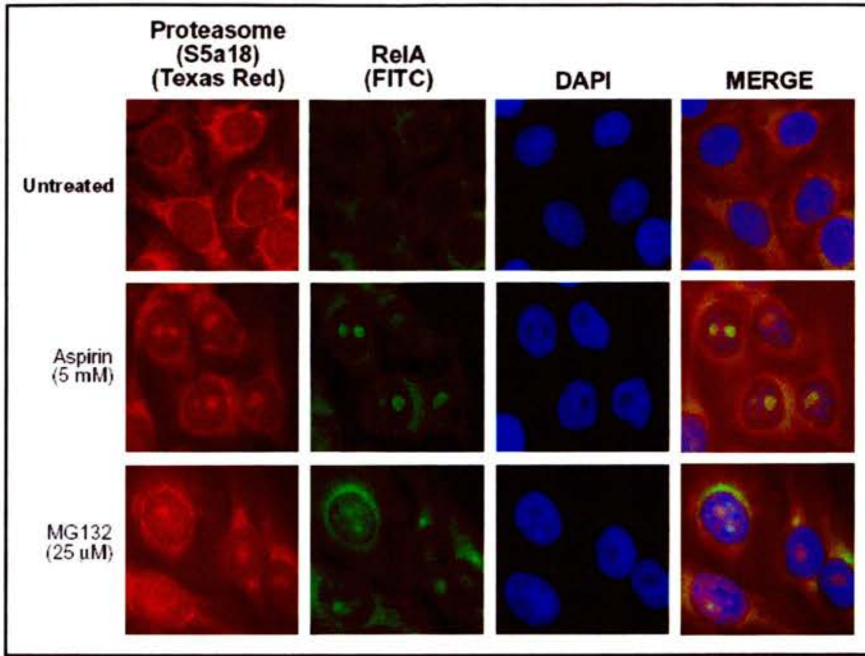



**Figure 6.4 – Aspirin and MG132 Reduce Total Proteasome Levels.** In three independent experiments, SW480 cells were treated overnight (16 hrs) with Aspirin (0, 5 mM) or MG132 (25 μM) then cytoplasmic extracts (15 μg) resolved by SDS PAGE on 10% polyacrylamide gels and anti-S5a-18 Western blot carried out. Actin was used as a loading control. Representative blots are shown.

these agents. The localisation of proteasomes in SW480 cells after 16 hrs treatment with aspirin or MG132 was examined by means of immunocytochemistry. Figure 6.5A demonstrates that in untreated control cells, proteasomes are located diffusely throughout the cytoplasm and nucleus. In cells treated with aspirin or MG132, there was little change in cytoplasmic staining of proteasomes or RelA but they were found to co-localise in nucleoli (distinct nuclear bodies, devoid of DAPI staining). It should be noted that in Figure 6.5A, the anti-proteasome antibody used was raised against the S5a-18 subunit of the proteasome. However, it was not possible to carry out dual staining of proteasomes and fibrillarin with that particular anti-S5a-18 antibody. Therefore, a proteasome antibody raised against a different subunit of the proteasome, the MTS4 subunit, but which did allow dual staining with anti-fibrillarin antibody and hence investigation of co-localisation of proteasomes and fibrillarin, was obtained from Dr Colin Gordon (MRC Human Genetics Unit, Edinburgh). It is noteworthy that proteasome staining is similar in both treated and untreated cells for both the S5a-18 and MTS4 antibodies (Figure 6.5A and 6.5B). Similar to the observation for β-catenin when cells were treated with aspirin or sulindac (see 5.2.2), fibrillarin seems to form a 'cap' around the proteasome (Figure 6.5B). As discussed for β-catenin, this could be due to disruption of the nucleolus in response to aspirin or MG132. However, the lack of DAPI staining in the region where proteasomes are sequestered in the nucleus strongly suggests that proteasomes localise to the nucleolus after treatment with aspirin and MG132. This data supports the hypothesis that RelA is subject to ubiquitin-mediated proteolysis in the nucleolus in response to both aspirin and MG132 treatment.

A




B

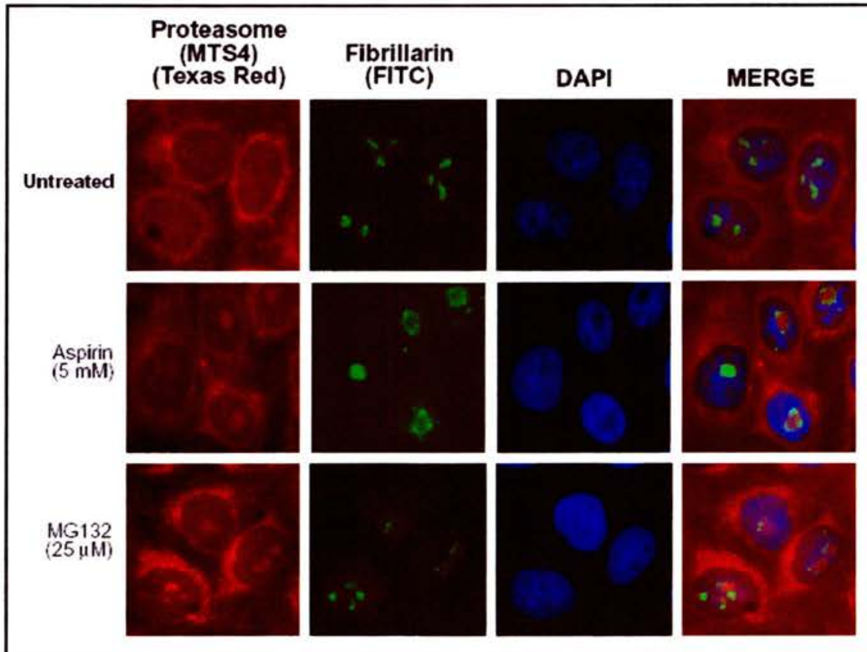



**Figure 6.5 – Aspirin and MG132 Induce Nucleolar Localisation of Proteasomes.** SW480 cells were treated, in three independent experiments, for 16 hrs with Aspirin (5 mM), MG132 (25 µM) or untreated (control). **(A)** Representative micrographs (63 x) illustrating localisation of RelA and proteasomes (S5a-18) are shown. **(B)** Representative micrographs (63 x) illustrating localisation of proteasomes (MTS4) and the nucleolar protein, fibrillarin, are shown.

### 6.2.6 Aspirin Mediates a Reduction in Acetylated RelA

RelA is subject to regulation by acetylation (Quivy and Van Lint, 2004) and recent unpublished work in the host laboratory has demonstrated that acetylation is important for the apoptotic response to aspirin in SW480 colorectal cancer cells. In particular, it was shown that the histone deacetylase inhibitor (HDACi), TSA, blocks aspirin effects on nucleolar sequestration of RelA, repression of NF-κB driven transcription and apoptosis. Furthermore, lysines 122 and 123 of RelA were shown to be important in this response. The next research strand was therefore to determine whether aspirin has an effect on the acetylation status of RelA. To that end, SW480 cells were treated for 16 hrs with aspirin and immunoprecipitation carried out using an anti-RelA antibody. Western blot analysis of the immunoprecipitated samples with antibodies to acetylated lysine established that there is an increase in total acetylation after aspirin treatment when compared with the untreated control (compare tracks 1 and 2 in Figure 6.6, particularly the areas highlighted by red arrows). It is well established that aspirin can acetylate and thus irreversibly inactivate both COX-1 and COX-2 (Kalgutkar *et al.*, 1998). Given that aspirin can directly acetylate proteins such as COX, the observed increase in total protein acetylation after aspirin treatment was not unexpected. However, in marked contrast from the expected findings, there was a significant decrease in acetylated RelA after aspirin treatment (compare band for acetylated RelA in tracks 5 and 6 of Figure 6.6). This data strongly suggests that RelA is deacetylated in response to aspirin treatment.

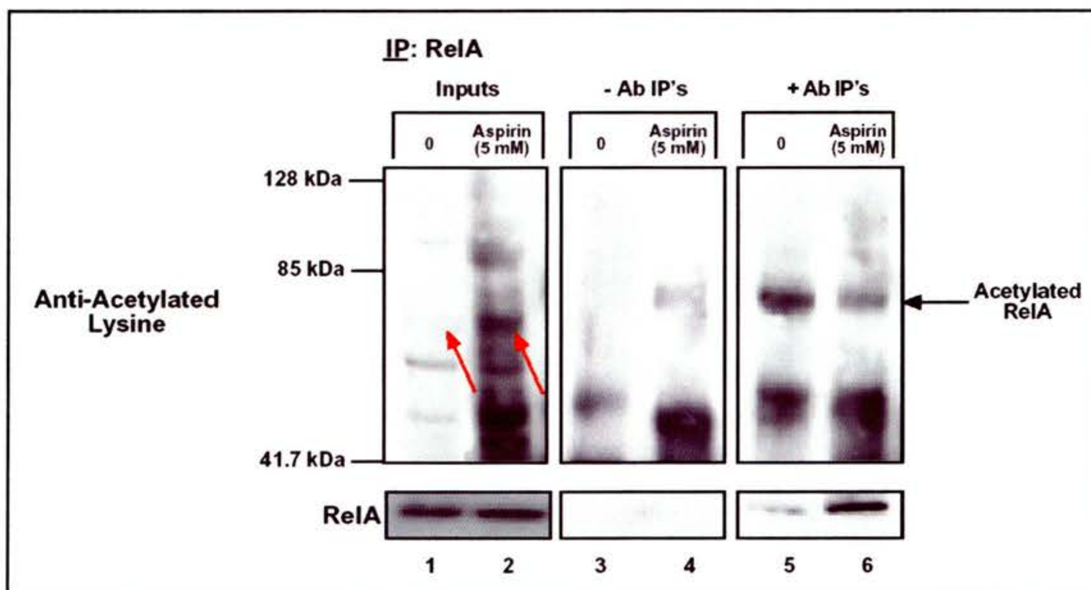



**Figure 6.6 – Aspirin Induces a Decrease in Acetylated RelA.** In three independent experiments, SW480 cells were treated for 16 hrs with Aspirin (0, 5 mM) then whole cell extracts (500 μg) immunoprecipitated (IP) using anti-RelA (rabbit) antibody (+ Ab) or no antibody (- Ab) as a control. Inputs (30 μg) and immunoprecipitates were resolved by SDS PAGE on 8% polyacrylamide gels and Western blot analysis using anti-acetylated lysine (mouse) antibody carried out. Blots were stripped and re-probed with anti-RelA (mouse) antibody. Representative blots are shown. Black arrow indicates the bands for acetylated RelA; Red arrows highlight areas of total protein acetylation described in the text.

### 6.2.7 MG132 Induces Nucleolar Localisation of β-catenin

In chapter 5, data are presented which suggest that interaction of RelA and β-catenin is implicated in nucleolar localisation of these proteins. Therefore, given that MG132 causes RelA to localise to the nucleolus, the final aim of the work presented in this chapter was to determine whether interaction of RelA and β-catenin occurs in response to MG132 and to establish the significance of this in relation to nucleolar localisation. Initially, immunocytochemistry was used to examine the subcellular localisation of β-catenin in SW480 cells treated for 16 hrs with MG132 (Figure 6.7). It was found that β-catenin is predominantly cytoplasmic, concentrated at the cell membrane in untreated cells but localises to distinct nuclear bodies corresponding to areas devoid of DAPI stain, which have previously been shown to be the nucleolus, after treatment with MG132. Similar to earlier observations, the nucleolar marker, fibrillarin, forms a 'cap' around β-catenin in cells treated with MG132, which could be explained by this agent causing disruption to the nucleolus.

### 6.2.8 MG132 Does not Induce Repression of TCF Transcriptional Activity

Having observed that both β-catenin and RelA localise to the nucleolus after treatment with MG132, the effects of MG132 on β-catenin/TCF-driven transcription were next studied. SW480 cells were transiently transfected with the synthetic

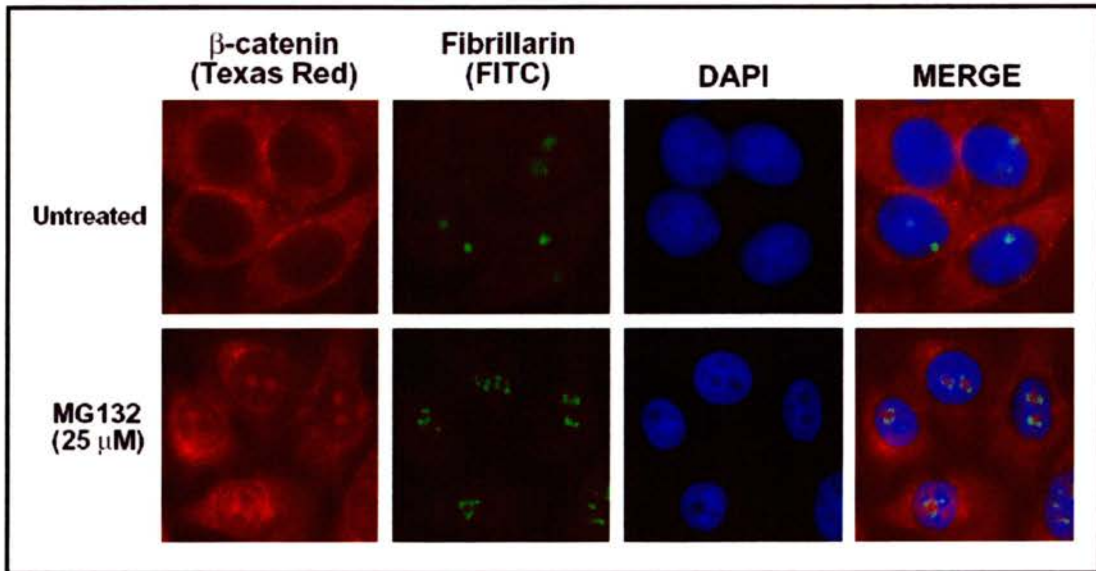



**Figure 6.7 – MG132 Induces Nucleolar Translocation of β-catenin.** SW480 cells were treated, in three independent experiments, for 16 hrs with MG132 (25 μM) or untreated (control). Representative micrographs (63 x) illustrating localisation of β-catenin and the nucleolar protein, fibrillarin, are shown.

TOPflash or FOPflash TCF reporter plasmids along with the pCMVβ control plasmid, then treated with MG132 for 16 hrs. In contrast to the observed repression of NF-κB-driven transcription (Figure 6.2), the results presented in Figure 6.8 indicate that MG132 has no effect on the level of relative β/catenin/TCF-driven luciferase activity when compared to the untreated control. It is important to state here again that problems with the pTOPFlash and pFOPFlash constructs were encountered when investigating the effects of NSAIDs on β-catenin/TCF-driven transcription (see section 5.2.3). Therefore, further study beyond the scope for this thesis regarding the effects of MG132 on β-catenin/TCF-driven transcription may be justified.

### 6.2.9 RelA and β-catenin Co-localise in the Nucleolus After MG132 Treatment

The discovery that both RelA and β-catenin localise to the nucleolus in response to treatment with MG132 strongly suggested that these proteins would co-localise there in response to this agent. To investigate this hypothesis, immunocytochemistry was employed to detect RelA and β-catenin in SW480 cells that had been treated overnight (16 hrs) with MG132 prior to harvesting. Figure 6.9 demonstrates that RelA and β-catenin do in fact co-localise within nucleoli after MG132 treatment in a similar manner to treatment with NSAIDs. This data indicates that RelA and β-catenin could interact in the nucleolus upon proteasome inhibition.

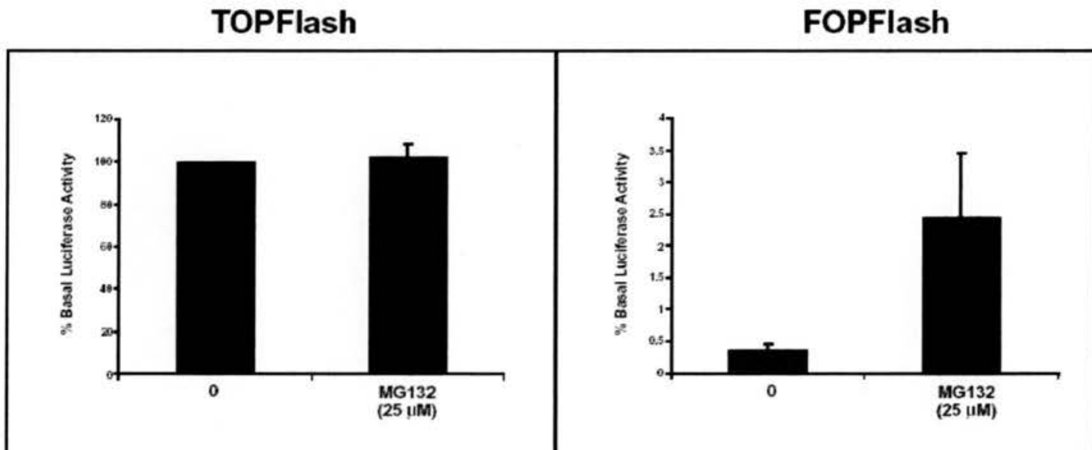



**Figure 6.8 – MG132 has no Effect on β-catenin/TCF-driven Transcriptional Activity.**

SW480 cells were transiently transfected with the synthetic β-catenin/TCF transcriptional reporter constructs, pTOPFlash or pFOPFlash, and the pCMVβ control plasmid, then subsequently treated with MG132 (0, 25 μM). Luciferase assays were carried out to measure β-catenin/TCF transcriptional activity and β-galactosidase assays were used to normalise these results for transfection efficiency. % transcriptional activity was calculated relative to non-treated controls. FOPFlash activity is expressed relative to TOPFlash activity. Results presented are the mean of three independent experiments (+/- standard deviation).

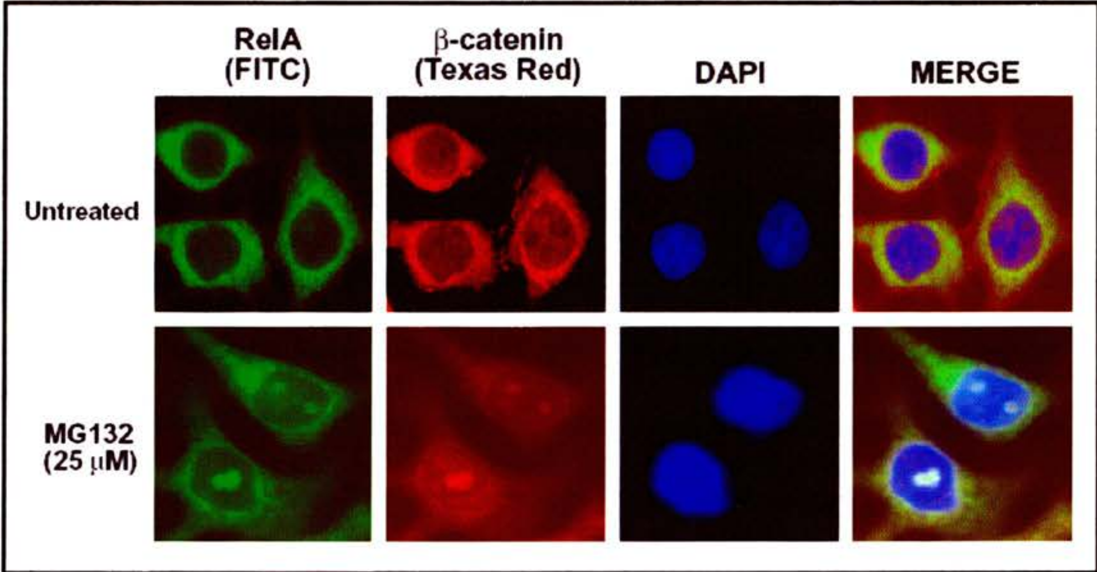



**Figure 6.9** – **RelA and β-catenin Co-localise in the Nucleolus after MG132 Treatment.**

SW480 cells were treated, in three independent experiments, for 16 hrs with MG132 (25 μM) or untreated (control). Representative micrographs (63 x) illustrating localisation of RelA and β-catenin are shown.

Moreover, the data demonstrates similarities of the two pathways in response to MG132.

## 6.3 Discussion

It has recently been demonstrated by the host laboratory that RelA accumulates in the nucleolus in response to aspirin treatment (Stark and Dunlop, 2005). Furthermore, it was established in Chapter 3 that, like aspirin, the NSAIDs sulindac, sulindac sulfone and indomethacin induce nucleolar sequestration of RelA. Although a nucleolar localisation motif at the N-terminus of RelA was previously identified (Stark and Dunlop, 2005), the precise mechanism of nucleolar translocation of RelA and function of RelA in the nucleolus remain to be determined. It is increasingly apparent that post-translational modifications, including acetylation and ubiquitination, can regulate nuclear NF-κB activity and so the major objective of this chapter was to investigate the significance of such modifications in nucleolar sequestration of RelA.

Initially, the main focus was to consider the role of ubiquitination of RelA and so the effect of MG132 on RelA localisation and NF-κB transcriptional activity was investigated. Figure 6.1 and Figure 6.2 demonstrate that MG132, like NSAIDs, causes nucleolar sequestration of RelA and repression of NF-κB transcriptional activity. This is the first study to demonstrate nucleolar localisation of RelA and repression of NF-κB transcriptional activity after proteasome inhibition, but it should be emphasized that the precise mechanisms still remain to be elucidated. One

possibility is that MG132 activates the NF-κB pathway in a similar manner to NSAIDs. Subsequent translocation of RelA to the nucleolus could then mediate repression of basal NF-κB transcriptional activity. There is conflicting evidence from the literature on whether proteasome inhibitors inhibit (Kisselev and Goldberg, 2001; Goldberg and Rock, 2002) or activate (Ferrari *et al.*, 1997; Németh *et al.*, 2004) the NF-κB pathway. However, the cellular effects of MG132 with respect to NF-κB are complex because proteasome inhibitors also block IκBα degradation (Kisselev and Goldberg, 2001; Goldberg and Rock, 2002) and so activation of NF-κB by MG132 may involve a distinct pathway. Moreover, by blocking IκBα turnover, nuclear translocation of RelA would also be blocked by MG132. Hence, repression of NF-κB activity could be caused by a reduction in cytoplasmic to nuclear shuttling of RelA. Another more speculative explanation could be that RelA is normally subject to ubiquitin-mediated proteolysis in the nucleolus and that by inhibiting the proteasome, there is an accumulation of ubiquitinated RelA there. As this proposed mechanism would not require activation of the NF-κB pathway by MG132, this is the favoured hypothesis.

To further investigate the hypothesis that RelA is subject to ubiquitin-mediated proteolysis in the nucleolus, the ubiquitin status of RelA after MG132 and aspirin treatment was examined. In keeping with an observed general increase in protein ubiquitination in response to MG132 and aspirin, it was discovered that both of these agents caused an increase in ubiquitinated RelA (Figure 6.3). Although far from conclusive on their own, these findings provide support for the theory that RelA is normally ubiquitinated and degraded. To speculate further, one possibility may be that aspirin behaves like the proteasome inhibitor to block ubiquitin-mediated

proteolysis of RelA. Hence, it could be conceived that aspirin causes a reduction in proteasome activity and/or a reduction in proteasome levels. In order to try and tackle the question of whether aspirin works in a similar manner to the proteasome inhibitor, the total cellular levels of proteasomes in response to aspirin and MG132 were next studied and a significant reduction in total cellular proteasome levels after both aspirin and MG132 treatment was found (Figure 6.4). Again, while accepting that this experiment provides only circumstantial evidence on its own, the data nonetheless suggests that aspirin behaves in a similar manner to MG132 in terms of reducing proteasome levels and hence provides further support for the hypothesis that aspirin acts like the proteasome inhibitor to cause an accumulation of ubiquitinated RelA.

Since the completion of experimental work presented here, a study published within the last month has reported that aspirin induces apoptosis through the inhibition of proteasome function (Dikshit *et al.*, 2006). In that study, treatment of Neuro 2a cells with aspirin led to a dose- and time-dependent decrease in proteasome activity and an increase in accumulation of ubiquitinated proteins in the cells. However, aspirin did not affect the protease activity of purified 20S proteasomes, leading the authors to propose that aspirin-induced proteasomal inhibition is mediated indirectly, perhaps by inhibiting expression of proteasome subunits. The effects of aspirin on proteasome activity were found to correlate with aspirin-induced cell death. Significantly, aspirin was found to result in an increase in the half life of various intracellular proteasome substrates, for example p53 and $p27^{kip1}$. Finally, the authors reported changes in mitochondrial membrane potential in response to aspirin, in particular release of cytochrome *c* and activation of caspase-9 and -3, which could

be the result of proteasome dysfunction. Collectively, the data from that study provides substantial evidence to support the observations here that aspirin causes a subtle increase in protein ubiquitination and significant increase in ubiquitination of RelA. In particular, the published findings that aspirin causes an increase in the half life of various substrates supports the notion that aspirin could affect the stability and/or turnover of RelA. Moreover, the discovery here that aspirin causes a decrease in proteasome levels is in keeping with the hypothesis from the above study that aspirin-induced proteasomal inhibition is mediated indirectly.

The conjugation of substrates with ubiquitin involves the successive action of three classes of enzymes, the E1 ubiquitin activating enzymes, the E2 ubiquitin-conjugating enzymes (Ubc), and the E3 ubiquitin-ligases (Ubl) [reviewed in (Roos-Mattjus and Sistonen, 2004; Mani and Gelmann, 2005; Zhou, 2005; Elsasser and Finley, 2005)]. It is now evident that components of the ubiquitin system, in particular proteasomes, are not evenly distributed throughout the cell and that proteolysis of some particular proteins only occurs in distinct cellular compartments (Wójcik and DeMartino, 2003; Lenk and Sommer, 2000), suggesting that proteasomes could scan for their substrates. In particular, there is a growing body of evidence that the nucleolus could be a site for proteasome-mediated degradation of certain proteins. Cellular levels of the transcriptional regulator, c-Myc are known to be regulated by the ubiquitin/26S proteasome pathway (Ciechanover *et al.*, 1991). It has previously been demonstrated that c-Myc shuttles between the cytoplasm and nucleus, raising the possibility that c-Myc could be degraded in either compartment (Arabi *et al.*, 2003). Moreover, it was discovered that by overexpressing c-Myc or elevating levels of c-Myc using the proteasome inhibitor, MG132, c-Myc and

proteasomes accumulate at the nucleolus, thus leading the authors to propose that c-Myc is negatively regulated by ubiquitin-mediated proteolysis in the nucleolus. Subsequent studies have revealed that a nucleolar isoform of the Fbw7 ubiquitin ligase regulates c-Myc (Welcker *et al.*, 2004). c-Myc has also been observed to associate with ribosomal DNA and activate RNA polymerase 1 transcription (Arabi *et al.*, 2005; Grandori *et al.*, 2005). Another study (Song and Wu, 2005) recently documented that Survivin-deltaEx3, a splice variant of the inhibitor of apoptosis protein, Survivin (SVV), localises to the nucleolus when ectopically expressed in transfected cells or upon treatment with proteasome inhibitors. In addition, a novel nucleolar localisation signal and degradation signal in SVV-deltaEx3 were identified and these were found to be required for the anti-apoptotic function of the protein. Furthermore, proteasome inhibitors greatly inhibited the degradation of SVV-delta-Ex3 but caused polyubiquitination of SVV-deltaEx3 and fusion of heterologous proteins, such as TAT-PTD or p14ARF, to the putative degradation signal resulted in significant degradation in the nucleolus (Song and Wu, 2005). Interestingly, it has been established that ARF interacts with and inhibits B23, a nucleolar endoribonuclease involved in maturation of 28S rRNA, by promoting its polyubiquitination and degradation (Itahana *et al.*, 2003). Proteins associated with the nuclear structure PODs, including PML, Sp100 and SUMO-1, have been reported to move to the nucleolus upon proteasome inhibition (Mattsson *et al.*, 2001). In that study, it was further discovered that movement of proteasomes to the nucleolus was associated with the movement of POD-associated proteins upon proteasome inhibition. Given that PODs have previously been proposed to be the site for proteasome-mediated degradation of certain ubiquitinated proteins (Everett, 2000;

Everett *et al.*, 1997; Wójcik and DeMartino, 2003; Smith *et al.*, 2004; Fabunmi *et al.*, 2001; Lallemand-Breitenbach *et al.*, 2001), this led the authors to propose that POD components and proteasomes specifically target the nucleolus when proteasome-dependent protein degradation is blocked (Mattsson *et al.*, 2001).

In light of the observations from the published studies outlined above, in particular the finding that proteasomes accumulate in the nucleolus following proteasme inhibition, the next focus of this study was to determine whether the localisation of proteasomes was affected by aspirin and MG132 treatment. Immunocytochemisy demonstrated that proteasomes localise to the nucleolus in response to both aspirin and MG132 treatment (Figure 6.5). Again, while accepting this data on its own is somewhat circumstantial, it nonetheless supports the hypothesis that RelA is subject to ubiquitin-mediated proteolysis in the nucleolus. Another interesting speculation arising from the observation that proteasomes localise to the nucleolus after aspirin and MG132 treatment is that they could carry RelA there. However, it will be important to determine the activity of proteasomes in the nucleolus as one might predict that they are non-functional given RelA accumulation. It would also be informative in the future to make use of another proteasome inhibitor, one that is structurally unrelated to MG132, or a structural anologue of MG132 that does not inhibit the proteasome in order to provide good controls for the specificity of the effects seen with MG132.

In addition to ubiquitination, it is well established that RelA is subject to regulation by acetylation (see Introduction of this Chapter and Chapter 1). However, there have been no studies to date looking at the effects of NSAIDs on acetylation of RelA. Recent unpublished work in the host laboratory has revealed that the HDACi,

TSA, can block aspirin-induced nucleolar translocation of RelA, repression of NF-κB activity and apoptosis. Furthermore, mutating lysines 122 and 123 (key acetylation sites of RelA) to arginines blocks aspirin-mediated nucleolar translocation of RelA and apoptosis. Given that these data indicate that aspirin effects, in particular nucleolar sequestration of RelA, are subject to modulation by acetylation, the next part of this study was to investigate the role of acetylation of RelA in the response to aspirin. The data presented demonstrates that there is a decrease in acetylated RelA after aspirin treatment (Figure 6.6). Again, while recognising that this data on its own is not conclusive, it nonetheless provides further support for the notion that there is a role for acetylation in the effects of aspirin. It would be of particular interest in the future to determine whether this effect is specific to aspirin or represents a general effect of NSAIDs. To that end, an important area of future investigation would be to establish whether the other NSAIDs used in this study also cause a reduction in acetylated RelA. Furthermore, a good control might be to examine the effects an HDACi, such as TSA, as this would have have the same general effect as aspirin in terms of increasing total protein acetylation. Identification of the specific lysine residues targeted by aspirin would also be of significance to any future studies, although Lysines 122 and 123 would be key candidates in light of the recent unpublished observations in the host laboratory mentioned above.

This is the first study to demonstrate an effect on the acetylation status of RelA in response to aspirin treatment. One possible explanation for the observed decrease in acetylated RelA after aspirin treatment could be that there is an increase in association of RelA with HDACs. It is noteworthy that such an association could

also be responsible, at least in part, for repression of NF-κB transcriptional activity in response to aspirin. The members of the classical HDAC family fall into two distinct phylogenetic classes, namely class I and class II [reviewed in (de Ruijter *et al.*, 2003)] based on homology to the yeast (*S. cerevisiae*) proteins RPD3 and HDA1 respectively. Class I HDACs include HDAC 1, 2, 3 and 8 whereas Class II HDACs include HDAC 4,5,6,7,9 and 10. A large body of evidence points to a role for class I HDACs in the association with co-repressor complexes to direct gene-specific transcriptional repression (Gray and Ekström, 2001). Whereas class I HDACs are thought to be expressed in most cell types, studies to date have documented a more restricted expression pattern of class II HDACs and so point to a role for class II HDACs in cellular differentiation and developmental processes (de Ruijter *et al.*, 2003). Therefore, the most likely candidate HDACs for interaction with RelA after aspirin treatment would be members of the class I HDACs, such as HDAC1, 2 and 3.

A recent study (Campbell *et al.*, 2004) reported that repression of anti-apoptotic NF-κB target genes by the atypical stimuli UV-C and doxorubicin is mediated by an increase in association of RelA with HDAC1, 2 and 3, supporting the notion that class I HDACs could be implicated in the response to aspirin. Moreover, another study from the same group demonstrated that the nucleolar protein $p14^{ARF}$ can regulate the transactivation function of RelA and inhibit both NF-κB-driven transcription and anti-apoptotic activity by inducing association of RelA with HDAC1 (Rocha *et al.*, 2003). To speculate further, interaction of RelA with HDACs after aspirin treatment may therefore mediate binding of proteins, such as $p14^{ARF}$, which could be important co-factors for nucleolar translocation of RelA. However, further research is clearly required to ascertain which, if any, HDACs interact with

RelA after aspirin treatment and whether or not such an interaction contributes to transcriptional repression of NF-κB, localisation of RelA to the nucleolus or binding of co-factors that are involved in these processes.

There is a growing body of evidence that acetylation-dependent mechanisms can control protein stability, although these are very complex and not fully understood. The stability of many proteins, including p53 and E2F-1, is known to increase after the acetylation of specific lysine residues in these proteins [reviewed in (Caron *et al.*, 2005)]. Several studies have also documented an accumulation of ubiquitin-protein conjugates in response to HDAC inhibition [reviewed in (Caron *et al.*, 2005)]. A very recent report (Alao *et al.*, 2006) demonstrated that the HDAC inhibitor, TSA, induces ubiquitin-dependent Cyclin D1 degradation in MCF-7 breast cancer cells. Similarly, it has been demonstrated that ubiquitin-independent proteasomal degradation of hypoxia-inducible factor-1α (HIF-1α) occurs in response to HDAC inhibition (Kong *et al.*, 2006). The treatment of cells with HDAC inhibitors has also been shown to directly affect the proteasome, either by modulating the concentration of its subunits or by affecting its activity (Caron *et al.*, 2005). Interestingly, it was recently established that HDAC inhibition prevents NF-κB activation by suppressing proteasome activity (Place *et al.*, 2005). In that study, butyrate and TSA were found to suppress TNF-α-induced NF-κB activation by preventing the proteasome-dependent degradation of IκBα. By down-regulating the expression of three catalytic proteasome subunits, β-5, β-1 and β-2, the resulting decrease in global proteasome activity led to stabilisation of IκBα after TNF-α stimulation (Place *et al.*, 2005). In relation to this study, it could therefore be postulated that acetylation might be another important mechanism for regulation of

RelA stability in response to NSAIDs. To speculate further, acetylation could even be implicated in controlling protein stability through influencing ubiquitin modifications and targeting of specific proteins, including RelA, for destruction in the proteasome. These would be very exciting areas for future research.

In Chapter 5, interaction of RelA and β-catenin was shown to be important for nucleolar sequestration of these proteins in response to NSAIDs and so the final focus of investigation was to establish whether such an interaction was also significant for the effects of MG132. Initially, the effects of MG132 on localisation of β-catenin and β-catenin/TCF-driven transcriptional activity was studied. Like RelA, β-catenin was found to localise to the nucleolus after treatment with MG132 (Figure 6.7). This is the first study to demonstrate nucleolar localisation of β-catenin in response to proteasome inhibition. It is well established that β-catenin is subject to ubiquitination and subsequent degradation by the proteasome pathway (Lustig and Behrens, 2003). Therefore, it could be postulated that under normal conditions and in an analogous manner to RelA, the nuclear pool of β-catenin is subject to degradation in the nucleolus. One could further surmise that by behaving like the proteasome inhibitor, MG132, NSAIDs block degradation of β-catenin in the nucleolus and hence cause its accumulation there. Although both RelA and β-catenin were found to localise to the nucleolus in response to treatment with MG132, in contrast to the observed repression of NF-κB transcriptional activity, MG132 was discovered to have little effect on TCF/β-catenin-driven transcriptional activity (Figure 6.8). However, it should again be emphasized that technical difficulties were encountered using the pTOPFlash and pFOPFlash constructs when studying the effects of NSAIDs on TCF/β-catenin-driven transcriptional activity and so further

investigation, beyond the scope of this thesis, into the effects of MG132 on TCF/β-catenin-driven transcriptional activity may be warranted.

The discovery that β-catenin and RelA both localise to the nucleolus after treatment with MG132 suggested that these proteins could interact there in response to this agent. The observation that RelA and β-catenin do indeed co-localise in the nucleolus in response to MG132 (Figure 6.9) provides support for this notion. To speculate further, ubiquitination could be implicated in the sequestration and potential interaction of RelA and β-catenin in the nucleolus, although the significance and mechanisms of such an interaction remain to be elucidated. It is noteworthy that IκBα and β-catenin interact with the same E3 ubiquitin ligase, β-TrCP (Maniatis, 1999), and so ubiquitination may be an important common regulatory mechanism to both the NF-κB and Wnt/β-catenin pathways and could therefore represent a key point of cross-regulation. There is also a growing body of evidence that, like RelA, β-catenin, is subject to regulation by acetylation (Wolf *et al.*, 2002; Wei *et al.*, 2003; Lévy *et al.*, 2004; Labalette *et al.*, 2004). It could therefore be postulated that acetylation of β-catenin might also be implicated in the sequestration and interaction of RelA and β-catenin in the nucleolus and hence could signify another point of cross-regulation of the NF-κB and Wnt/β-catenin pathways. Moreover, it could also be conceived that post-translational modifications of RelA and/or β-catenin, such as ubiquitination and acetylation, may serve to allow binding of any additional factors required for interaction of RelA and β-catenin.

While appreciating that it is far from conclusive, the data presented in this chapter suggests that ubiquitination and acetylation of RelA are both important in the mechanism and function of RelA sequestration in the nucleolus after aspirin

treatment. In particular, the data provides support for the favoured hypothesis that RelA is subject to ubiquitin-mediated proteolysis in the nucleolus under normal circumstances. Specifically, an interesting possibility arising from the data is that, by behaving like the proteasome inhibitor to reduce proteasome activity, aspirin leads to an accumulation of ubiquitinated RelA in the nucleolus and this is supported by a very recent report (Dikshit *et al*, 2006). In keeping with previous observations from the host laboratory that acetylation is important in the response to aspirin, the discovery that RelA acetylation was reduced after aspirin treatment provides further evidence to support the preferred theory that aspirin mediates an increased interaction of RelA with HDACs. In turn, such an interaction could, at least in part, account for the observed transcriptional repression of NF-κB after aspirin treatment and might serve to allow binding of a co-factor, such as $p14^{ARF}$, that could carry RelA to the nucleolus. It is possible that β-catenin is also subject to regulation by ubiquitination and/or acetylation in response to NSAIDs. Hence, it could be further surmised that post-translational modifications may implicated in interaction of RelA and β-catenin after NSAID treatment and for this reason, might represent important points of cross-regulation of the NF-κB and Wnt/β-catenin pathways. Moreover, the nucleolus could represent a key site for such cross-talk between the NF-κB and Wnt/β-catenin pathways.

# Chapter 7 – Discussion

Collectively, the data presented in this thesis shows that activation of the NF-κB pathway and nucleolar sequestration of RelA are critical for the anti-tumour effects of all of the NSAIDs studied. However the upstream mechanism of activation differs between aspirin and the other NSAIDs examined. It is also demonstrated in this thesis that NSAIDs modulate β-catenin signaling and that the nucleolus could be a key site for cross-regulation between the NF-κB and β-catenin pathways. Such cross-talk might represent an important regulatory mechanism in the apoptotic response to NSAIDs. Moreover, post-translational modifications, specifically ubiquitination and acetylation, of RelA may be important for nucleolar shuttling and induction of apoptosis in response to NSAIDs. A proposed model, which summarises the findings of this thesis and outlines the pathways and modifications utilised by the panel of NSAIDs studied in this thesis, is shown in Figure 7.1. This model, and the implications of the data presented in this thesis in terms of the direction of future research, will be the subject of this discussion.

## 7.1 Nucleolar Sequestration of RelA is a Common Mechanism by Which NSAIDs Induce Apoptosis

Previous work published from the host laboratory (Stark and Dunlop, 2005) demonstrated that nucleolar sequestration of RelA is causally involved in aspirin-

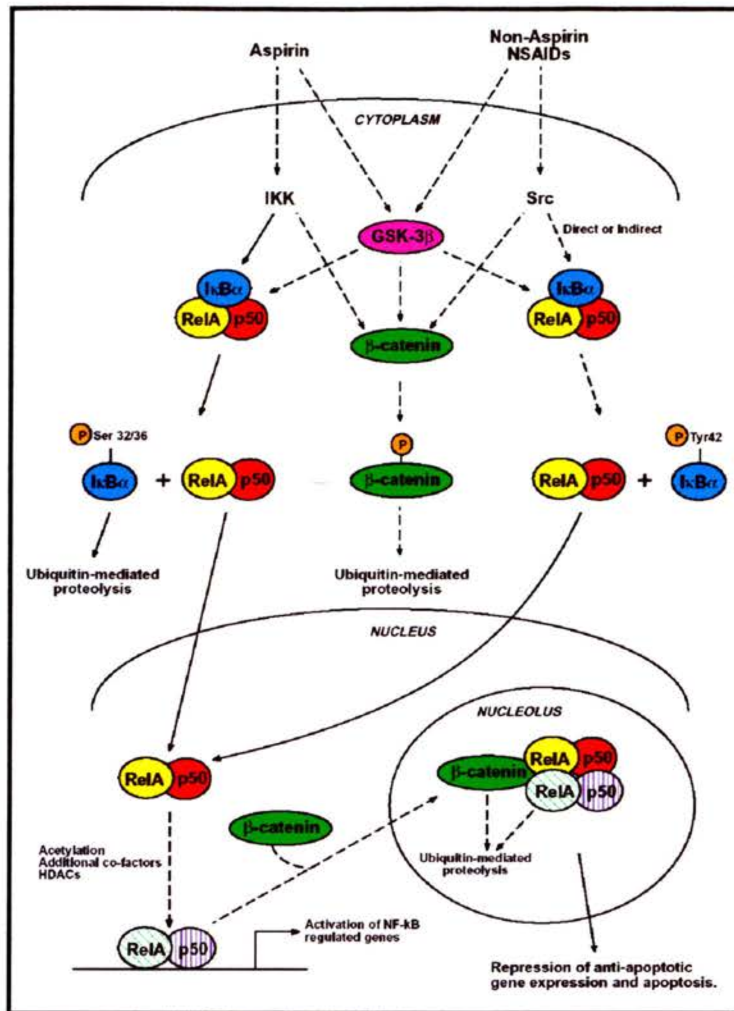



**Figure 7.1 – Proposed Model for Mechanism of Apoptosis Induced by NSAIDs.** Aspirin activates NF-κB by the 'classical' pathway, as previously described (Stark *et al.*, 2001), whereas non-aspirin NSAIDs are predicted to induce direct or indirect phosphorylation of IκBα on tyrosine 42 by c-Src and this tyrosine phosphorylated IκBα would not be degraded. Phosphorylation of IκBα on serine or tyrosine in response to NSAIDs subsequently results in unmasking of the nuclear localisation signal on RelA and translocation of NF-κB to the nucleus. Once in the nucleus, NSAID-induced NF-κB complexes are proposed to recruit and transport basal NF-κB complexes (shown in light blue and purple stripes) to the nucleolus, a process that could be regulated by acetylation, interaction with HDACs or additional co-factors. Once in the nucleolus, NF-κB complexes are sequestered away from target promoters, leading a decrease in transcription of anti-apoptotic genes and ultimately, apoptosis. Alongside activation of NF-κB, NSAIDs activate β-catenin. Cytoplasmic β-catenin is proposed to be subject to phosphorylation, perhaps mediated by GSK-3β or IKK, leading to degradation of β-catenin by the 26S proteasome. Nuclear β-catenin, however, is postulated to localise to the nucleolus, possibly mediated by association with RelA. β-catenin and/or RelA may be subject to ubiquitin-mediated proteolysis in the nucleolus. Points of potential cross-talk and/or incompletely defined pathways are indicated by dashed arrows.

induced repression of NF-κB-driven transcriptional activity and apoptosis. In keeping with these data, it was discovered in Chapter 3 that the non-aspirin NSAIDs, sulindac, sulindac sulfone and indomethacin, cause nucleolar localisation of RelA. Significantly, sequestration of RelA in the nucleolus in response to non-aspirin NSAIDs was found to be associated with repression of NF-κB-driven transcriptional activity. Furthermore, by overexpressing a dominant negative RelA construct deleted for the NoLS [RelA(Δ27-30)], nucleolar accumulation of RelA in response to treatment with sulindac, sulindac sulfone and indomethacin was shown to be absolutely required for apoptosis induced by these agents. Taken together, these data strongly suggest that sequestration of RelA in the nucleolus is a common mechanism whereby NSAIDs as a class induce apoptosis.

As discussed in Chapter 3, the precise mechanisms for localisation of proteins to the nucleolus are not known. Any soluble molecule could, in principle, diffuse in and out of the nucleolus as there is no structural or functional evidence for the existence of a frontier separating this nuclear compartment from the surrounding nucleoplasm (Carmo-Fonseca *et al.*, 2000). However, nucleolar accumulation is generally mediated by the presence of a specific nucleolar targeting sequence (Scheer and Hock, 1999) or via molecular interaction with a nucleolar partner (Olson, 2004).

The host laboratory has previously reported that TNF and TRAIL cause RelA to be concentrated in the nucleoplasm. In contrast, the atypical pro-apoptotic stimuli, serum withdrawal and UV-C radiation, result in localisation of RelA in the nucleolus (Stark and Dunlop, 2005). It was therefore postulated that the upstream mechanism of activation of NF-κB could be implicated in nucleolar sequestration of RelA. However, although aspirin, sulindac, sulindac sulfone and indomethacin were all

found in this study to cause nucleolar accumulation of RelA, the mechanism of NF-κB activation differed between aspirin and the non-aspirin NSAIDs, suggesting that it is not the upstream mechanism of activation of NF-κB that influences the sub-nuclear localisation of RelA. In other words, there is a common final pathway. One possibility is that aspirin and other NSAIDs both induce a secondary event which is important in determining the sub-nuclear distribution of RelA. This hypothesis is supported by the published evidence for aspirin (Stark and Dunlop, 2005) and observations in Chapter 3 that the 4 amino acid NoLS is important in nucleolar sequestration of RelA in response to both aspirin and non-aspirin NSAIDs. Moreover, previous data established that de-novo protein synthesis is required for RelA to localise to the nucleolus (Stark and Dunlop, 2005).

There is evidence from the literature that β-catenin can regulate nuclear NF-κB function (see Introduction of Chapter 5). Data presented in Chapter 5 suggest that activation of β-catenin could be implicated in nucleolar sequestration of RelA in response to aspirin, based on the observation that inhibiting nucleolar sequestration of RelA, by overexpressing the NoLS deletion mutant, blocks nucleolar localisation of β-catenin in response to aspirin. These data indicate that RelA and β-catenin could interact, possibly at the NoLS, in response to aspirin and that such an interaction may be causally involved in accumulation of these proteins in the nucleolus in response to that agent. Previous studies have demonstrated an interaction between RelA and β-catenin and that β-catenin can regulate nuclear NF-κB and vice versa (Deng *et al.*, 2002; Masui *et al.*, 2002; Deng *et al.*, 2004). However, it was not possible to replicate these findings, most likely due to technical problems. On the other hand, it is also possible that the apparent lack of direct interaction is real in the cell systems

used here. Hence, it is feasible that nucleolar sequestration of RelA influences nucleolar localisation of β-catenin indirectly.

It is well documented that post-translational modifications, in particular ubiquitination, can regulate the nuclear activity of certain proteins, such as RelA and β-catenin. The data presented in Chapter 6 suggest that post-translational modification plays a role in nucleolar accumulation of RelA and/or β-catenin. In particular, it was found that **1**. MG132 mimics the effects of aspirin on localisation of both RelA and β-catenin. **2**. Aspirin and MG132 cause an increase in general protein ubiquitination and ubiquinated forms of RelA. **3**. Aspirin and MG132 cause a reduction in proteasome levels **4**. Components of the proteasome localise in the nucleolus in response to aspirin as well as to MG132. Based on these observations, it was hypothesised that nucleolar accumulation is involved in the regulation of the stability of RelA and/or β-catenin through ubiquitin-mediated targeting for destruction. As discussed in Chapter 6, it should be emphasized that a very recent paper published within the last month (Dikshit *et al*., 2006) provides significant support for the observed findings of this part of research. In particular, the authors report that aspirin causes a dose- and time-dependent decrease in proteasome activity and an increase in ubiquitinated proteins. Noteably, aspirin was found to increase the half life of various intracellular proteasome substrates, for example p53 and $p27^{kip1}$. In addition to the effects on cellular proteins, changes in mitochondrial membrane potential resulting from proteasome dysfunction, were proposed to mediate apoptosis in response to aspirin. Finally, the authors postulated that aspirin inhibits proteasome activity indirectly, which is in keeping with the discovery here that aspirin causes a reduction in proteasome levels.

There is now a growing body of evidence that proteolysis of particular proteins occurs within distinct cellular compartments, such as the nuclear structure PODs and the nucleolus. For example, c-Myc is thought to be subject to ubiquitin-mediated proteolysis in the nucleolus, based on the findings of several studies. The proteasome inhibitor, MG132, has been reported to cause c-Myc and proteasomes to accumulate at the nucleolus (Arabi *et al.*, 2003). Interestingly, in that study, overexpression of c-Myc was also found to cause c-Myc and proteasomes to localise to the nucleolus. Another study (Welcker *et al.*, 2004) reported that a nucleolar isoform of the Fbw7 ubiquitin ligase can regulate c-Myc.

Further work would be required to substantiate the speculative notion that RelA and/or β-catenin are subject to ubiquitin-mediated proteolysis in the nucleolus under normal circumstances and that aspirin can block this process. Such experiments would involve establishing whether aspirin increases levels of ubiquitinated β-catenin. In particular, it would be important to isolate proteins specifically from nucleoli to see if there is an increase in ubiquitinated RelA and/or β-catenin after aspirin treatment in that cellular fraction and to carry out *in vitro* ubiquitination assays for RelA and/or β-catenin to further characterise the exact process involved. Identification of the specific lysine residues of RelA and/or β-catenin that are modified would also be informative. A previous report (Ryo *et al.*, 2003) identified SOCS-1 as the putative E3 ubiquitin ligase for RelA and demonstrated that Pin1 inhibited SOCS-1-mediated proteolysis of RelA. It would therefore be interesting to investigate the role of SOCS-1 and Pin1 in ubiquitination of RelA and/or β-catenin after NSAID treatment. Finally, determining the activity of proteasomes in the nucleolus would be of great interest. If the favoured hypothesis is

correct, one would predict that proteasomes are non-functional after aspirin treatment, hence allowing accumulation of ubiquitinated RelA and/or β-catenin.

Acetylation is another post-translational modification that has been shown to regulate both RelA and β-catenin and hence might possibly modulate the sub-nuclear localisation of these proteins. There is substantial evidence that nuclear shuttling and the transcriptional and DNA binding activities of RelA are regulated by acetylation (Quivy and Van Lint, 2004; Schmitz *et al.*, 2004; Perkins and Gilmore, 2006). Recent unpublished data from the host laboratory has demonstrated that TSA, an inhibitor of HDACs, blocks nucleolar translocation of RelA, repression of NF-κB-driven transcription and apoptosis in response to aspirin. In Chapter 6 it was shown that there is a decrease in acetylated forms of RelA after treatment with aspirin. Collectively, these data provide support for the notion that acetylation of RelA is important in the response to aspirin.

As mentioned in Chapter 6, it is important to note that several studies have documented an accumulation of ubiquitin-protein conjugates in response to HDAC inhibition (Caron *et al.*, 2005). Moreover, HDAC inhibitors have been shown to directly affect the proteasome. In particular, a recent study (Place *et al.*, 2005) established that HDAC inhibition prevents NF-κB activation by suppressing proteasome activity. Therefore, in relation to the previous data from the host laboratory and the results presented here, it may be the case that TSA works by modulating aspirin effects on the proteasome, as opposed to inhibiting acetylation. This question could be addressed initially by studying the effects of TSA on the concentration of proteasome subunits and proteasome activity in response to aspirin.

It would then be of interest to determine whether TSA modulates the effects of aspirin on the acetylation and ubiquitination status of RelA.

Based on recent results published by the host laboratory, it was postulated that once in the nucleus, NF-κB complexes induced by aspirin recruit a specific co-factor to basal, promoter-bound NF-κB complexes, ultimately leading to transportation of all nuclear NF-κB complexes to the nucleolus (Stark and Dunlop, 2005). As discussed in Chapter 3, candidate co-factors include the nucleolar proteins, NFBP and B23, which have both recently been shown to interact with RelA (Sweet *et al.*, 2003; Dhar *et al.*, 2004), and the NF-κB regulator, NIK and NRF, because the function and mobility of these proteins respectively have been reported to be affected by nucleolar shuttling (Birbach *et al.*, 2004; Niedick *et al.*, 2004). A particularly strong candidate is ARF, a nucleolar protein that has recently been demonstrated to induce ATR- and Chk1-dependent phosphorylation of RelA at threonine 505, an event that is required for ARF-dependent repression of RelA transcriptional activity (Rocha et al., 2005). Specifically, ARF mediates repression of NF-κB-driven transcriptional activity by inducing association of RelA with HDAC1, an effect that is independent of MDM2 and p53 (Rocha *et al.*, 2003). It is noteworthy that aspirin-induced nuclear translocation of RelA and apoptosis has also previously been established to be independent of p53 status (Din *et al.*, 2005). In light of these published findings, the results presented here, and recent studies from the host laboratory which suggest that acetylation is important for aspirin-effects on the NF-κB pathway, it seems plausible that aspirin induces an association of RelA with HDACs. This could, at least in part, mediate repression of NF-κB transcriptional activity. Moreover, interaction of RelA with HDACs may allow binding of proteins,

such as ARF, which could be important co-factors for nucleolar translocation of RelA. Work is ongoing in the host laboratory to determine whether RelA interacts with HDACs after aspirin treatment and to identify co-factors that might be implicated in nucleolar localisation of RelA.

Taken together, the data discussed so far indicate that nucleolar sequestration of certain proteins, such as RelA and β-catenin, is important for NSAID-induced apoptosis. Moreover, given best efforts in the time available, the data goes some way to understanding the role and mechanisms of nucleolar localisation of RelA and β-catenin in the response to aspirin in particular.

## 7.2 c-Src is Required for Stimulation of the NF-κB Pathway and Apoptosis Mediated by Sulindac, Sulindac Sulfone and Indomethacin

It was of particular interest that there are differences in the mechanism by which aspirin and non-aspirin NSAIDs bring about activation of the NF-κB pathway. As discussed in Chapter 3, this raises the interesting possibility that structural differences could account for the discrepancy between aspirin and the other NSAIDs.

The data presented in Chapter 4 strongly implicate c-Src in the mechanism of activation of the NF-κB pathway by the panel of NSAIDs, based on the following observations: **1**. nuclear/nucleolar translocation of RelA in response to sulindac, sulindac sulfone and indomethacin, but not aspirin, can be abrogated by the c-Src inhibitor, PP2. **2**. c-Src is activated at 1-2 hrs in response to treatment with non-

aspirin NSAIDs. **3**. SW480 cells stably transfected with kinase dead c-Src (SW480-SrcKD) are resistant to sulindac-, sulindac sulfone- and indomethacin-induced repression of NF-κB activity and apoptosis whereas the equivalent control cells stably transfected with the pBpuro vector (SW480-pBpuro) behaved like parental SW480 cells in response to these NSAIDs. These data support previous observations that c-Src directly phosphorylates IκBα (Abu-Amer *et al.*, 1998; Fan *et al.*, 2003). Additional support for this hypothesis comes from the finding that c-Src can directly interact with IκBα (Abu-Amer *et al.*, 1998). For that reason, it would be important to establish, by co-immunoprecipitation studies, whether c-Src interacts with IκBα after NSAID treatment. It should also be emphasized that c-Src might not directly phosphorylate IκBα but instead could activate another kinase that phosphorylates IκBα. It is of particular interest that PI 3-kinase has been implicated in NF-κB-activation through the tyrosine phosphorylation-dependent pathway. Specifically, the regulatory subunit of PI 3-kinase, p85α, has previously been shown to associate with tyrosine phosphorylated IκBα (Béraud *et al.*, 1999; Kang *et al.*, 2003), and can interact with v-Src (Haefner *et al.*, 1995).

Despite extensive experimentation as part of the work presented in this thesis, it was not possible to show that tyrosine phosphorylation of IκBα and phosphorylation of IκBα by c-Src occur in response to to NSAIDs. It should be highlighted that positive controls using stimuli that have previously been reported to induce these responses could also not be shown to exert this effect. Thus, the experimental data are incomplete. However, it is also noteworthy that since the original report of tyrosine phosphorylation of IκBα, many groups have had difficulty detecting this phosphorylated form of IκBα (personal communication). Despite

trying to overcome the problem of detection by overexpressing IκBα in colorectal cancer cells, the tyrosine phosphorylated form of IκBα was still not discovered in response to sulindac. However, it should be pointed out that c-Src was not overexpressed in the same experiment and so, given further time, this would be an area worthy of further exploration as this approach was used in previously published work (Fan *et al.*, 2003). Another way to improve detection of tyrosine phosphorylated IκBα might be the development of a robust phosphospecific antibody.

If c-Src does not directly phosphorylate IκBα on tyrosine, it is possible that this kinase modulates NF-κB in another way so as to allow nuclear translocation of RelA without IκBα degradation. As discussed in Chapter 4, c-Src has previously been shown to target the IKK complex (Huang *et al.*, 2003b; Huang *et al.*, 2003a; Funakoshi-Tago *et al.*, 2005). However, these studies reported degradation of IκBα and so it is unlikely that c-Src activates IKK in response to NSAIDs. Direct phosphorylation of RelA itself is known to modulate nuclear NF-κB activity (Schmitz *et al.*, 2004; Chen and Greene, 2004; Perkins and Gilmore, 2006). Interestingly, NF-κB activation through tyrosine phosphorylation of both IκBα and RelA has recently been demonstrated in response to crystalline silica (Kang *et al.*, 2006 and 2003) and resveratrol (Pellegatta *et al.*, 2003), raising the intriguing possibility that tyrosine phosphorylation of RelA itself by c-Src could be implicated in the mechanism of activation of NF-κB by NSAIDs.

This is the first study to demonstrate activation of c-Src in response to NSAIDs and so, as mentioned in Chapter 4, an important area of future research would be determine the precise mechanism of activation of c-Src in response to

NSAIDs. Csk and protein tyrosine phosphatases regulate phosphorylation of c-Src at tyrosine 527 and hence the negative inhibition of phosphorylation of this residue (Playford and Schaller, 2004). Therefore, the phosphorylation status of residue tyrosine 527 of c-Src, the autoinhibitory phosphorylation site, should first be investigated in order to confirm the activation status of c-Src in response to NSAIDs. Assays for the activity of Csk and/or protein tyrosine phosphatases could then be carried out to establish whether these proteins are implicated in activation of c-Src by NSAIDs. Activation of FAK, a c-Src effector, is known to cause c-Src to adopt an active conformation via displacement of intramolecular SH-binding interactions (Frame, 2002) and so it would next be of interest to examine the role of this kinase further. Finally, given that c-Src is known to be activated by cell adhesion molecules such as integrins (Jones *et al.*, 2000; Westhoff *et al.*, 2004), it would subsequently be of value to look at the effects of NSAIDs on cell adhesion and integrin levels in order to see whether loss of cell adhesion in response to these drugs not only activates c-Src but is also implicated in the mechanism of apoptosis in response to these agents. Recent data from the host laboratory has found that p38, a member of the MAPK family, is rapidly activated after aspirin treatment and is involved in aspirin effects on NF-κB and apoptosis (Thoms *et al.*, 2004). Therefore, the role of p38 in non-aspirin NSAID-mediated effects on c-Src may also be worthy of future research.

Data presented here showing activation of c-Src in association with apoptosis of colorectal cancer cells would appear to contradict previous studies demonstrating that elevated levels of c-Src are associated with the malignant progression of colorectal cancer [reviewed in (Summy and Gallick, 2003)]. One report (Talamonti *et al.*, 1993) documented that c-Src activity is higher in primary colon tumours than

in adenomatous polyps and that the highest levels of c-Src activity were present in metastatic liver lesions. Furthermore, it has been shown that reduced expression of c-Src by an antisense approach can suppress the growth of HT29 colon carcinoma cells and that when these antisense transfected cells are injected into nude mice, very slow-growing tumours form (Staley *et al.*, 1997). Using the same approach, it was discovered that antisense-mediated downregulation of c-Src in HT29 cells resulted in decreased expression of VEGF, an important angiogenic factor thought to be associated with increased tumour cell motility (Ellis *et al.*, 1998). Although these data indicate that activation of c-Src is associated with progression of colon cancer, there is also evidence that activation of c-Src is associated with apoptosis. In particular, c-Src activation has been shown to regulate a form of detachment-induced apoptosis, anoikis, in human colon tumour cell lines (Windham *et al.*, 2002). In contrast to tumour progression, which would require constitutive activation of c-Src, induction of apoptosis may involve just a short burst of c-Src activity. Support for this hypothesis comes from the observation that c-Src activation occurs at 1-2 hrs treatment with sulindac, sulindac sulfone and indomethacin, but this response is terminated by 5 hrs. It is likely that upon a short, single burst, c-Src phosphorylates different downstream targets compared to when it is constitutively activated.

To summarise, the data from this section of work present exciting novel observations that the tyrosine kinase c-Src is activated in response to the NSAIDs sulindac, sulindac sulfone and indomethacin, and that this activation is implicated in stimulation of the NF-κB pathway and the mechanism of apoptosis in response to these agents. In Chapter 3 it was observed that IκBα is not degraded by sulindac, sulindac sulfone and indomethacin. It has previously been established that tyrosine

phosphorylation of IκBα can occur in the absence of its degradation (Imbert *et al.*, 1996). The data regarding c-Src is therefore in keeping with the notion of a role for tyrosine phosphorylation of IκBα in response to the non-aspirin NSAIDs used. Two published studies have reported that c-Src can directly phosphorylate IκBα (Abu-Amer *et al.*, 1998; Fan *et al.*, 2003). Hence, the favoured hypothesis is that c-Src phosphorylates IκBα in response to sulindac, sulindac sulfone and indomethacin, especially since c-Src is activated at 1-2 hrs upon treatment with these agents.

## 7.3 NSAIDs Modulate β-Catenin Signaling

In Chapter 5, it was discovered that NSAIDs induce degradation of cytoplasmic β-catenin, although the precise mechanisms for these effects still remain to be elucidated. As mentioned in Chaper 5, established upstream kinases of β-catenin include GSK-3β and IKK (Bienz, 2005; Lamberti *et al.*, 2001), making these very good candidates for future study. Interestingly, β-catenin has been shown to be phosphorylated by recombinant c-Src *in vitro* (Roura *et al.*, 1999). Furthermore, the authors also discovered that c-Src-mediated tyrosine phosphorylation of β-catenin decreases its binding to E-cadherin. A more recent study (Haraguchi *et al.*, 2004) demonstrated that v-Src can activate β-catenin/TCF-dependent transcription, an effect which could be partially abrogated by a dominant negative mutant of MAPK, suggesting that v-Src exerts its effect on β-catenin, at least in part, via the MAPK pathway. It should be emphasized that upstream kinases of β-catenin could represent important points of cross-regulation with the NF-κB pathway. For instance, GSK-3β

and IKK are established kinases of both the NF-κB and β-catenin pathways (Hoeflich *et al.*, 2000; Lamberti *et al.*, 2001).

In addition to investigating the role of upstream kinases of β-catenin, it would be of interest to examine the activity of β-TrCP in response to NSAIDs, as activation of this protein could account for the observed effects of NSAIDs on cytoplasmic β-catenin levels. Such an investigation would also be of significance in light of data presented in Chapter 5 which indicate that ubiquitination is important in the response to aspirin (discussed above). It is noteworthy that β-TrCP is a common ubiquitin ligase to both β-catenin and IκBα. Therefore, β-TrCP could also represent an important point of cross-regulation of the NF-κB and β-catenin pathways.

One possible explanation for the observations in Chapter 5 that cytoplasmic β-catenin is degraded and that β-catenin accumulates in the nucleolus in response to NSAID treatment is that there are two pools of β-catenin, a cytoplasmic pool and a nuclear pool. In such a model, the cytoplasmic β-catenin pool is subject to degradation, while the nuclear pool localises to the nucleolus after treatment with NSAIDs. Previous reports have shown that NSAIDs can target the nuclear activity of β-catenin in the absence of any effect on total β-catenin levels. One study (Dihlmann *et al.*, 2001) demonstrated that aspirin and indomethacin can inhibit β-catenin/TCF driven transcriptional activity without affecting endogenous β-catenin levels. Another study (Boon *et al.*, 2004) established that sulindac treatment specifically targets nuclear β-catenin expression in the adenomas of FAP patients. Although there is a body of data regarding the effects on NSAIDs on total cellular β-catenin levels, there is, unfortunately, very little data regarding the effects of NSAIDs on cytoplasmic β-catenin levels. One study has reported a reduction in cytoplasmic β-

catenin levels in response to sulindac sulfone (Thompson *et al.*, 2000), but the authors also found a reduction in nuclear β-catenin levels in response to that agent. Interestingly, β-catenin has been shown to be processed by caspase-3 (Webb *et al.*, 1999), suggesting that reduced β-catenin levels observed here could be a consequence of NSAID-induced apoptosis. However, preliminary data from time course studies in the host laboratory indicated that the reduction in cytoplasmic β-catenin protein levels caused by aspirin treatment occurs before aspirin-induced apoptosis. Therefore it seems unlikely that NSAID-mediated reduction in cytoplasmic β-catenin levels is a consequence of apoptosis in response to these agents.

The model that NSAIDs target different cellular pools of β-catenin could be tested by examining cytoplasmic β-catenin levels in cells transfected with the dominant negative RelA construct deleted for the NoLS which do not exhibit nucleolar sequestration of β-catenin. If NSAID-induced degradation of cytoplasmic β-catenin and nucleolar localisation of β-catenin are distinct events, it would be expected that reduction in cytoplasmic β-catenin levels could still take place if nucleolar translocation of β-catenin is inhibited. Another possible experiment to establish whether NSAID-mediated degradation of cytoplasmic β-catenin and nucleolar localisation of β-catenin are distinct events would be to investigate the effects of inhibiting β-catenin degradation, by overexpressing a mutant form of β-catenin that cannot be degraded.

Chapter 5 presents novel observations that β-catenin translocates to the nucleolus in response to NSAIDs. Previous studies have shown that NSAIDs modulate β-catenin protein levels and mediate a reduction in TCF-driven

transcriptional activity (Rice *et al.*, 2003; Dihlmann *et al.*, 2001 and 2003; Gardner *et al.*, 2004). However, this is the first study to demonstrate nucleolar localisation of β-catenin in response to NSAIDs or any other agents. It may be speculated that nucleolar localisation of β-catenin is causally involved in downregulation of TCF-driven transcriptional activity in an analogous fashion to RelA/NF-κB. To test this hypothesis, TCF reporter assays could be performed in cells expressing RelA deleted for the NoLS of RelA which do not exhibit nucleolar translocation of β-catenin (see Chapter 5). However, it is important to note that repression of TCF-driven transcription was not specific in response to NSAIDs as a decrease in both TOPFlash and FOPFlash activity was observed in response to NSAIDs (Chapter 5). Despite best efforts in the time available, the reasons for the decrease in FOPFlash activity were unclear, especially since published data suggests that NSAIDs have little effect on activity from this construct (Dihlmann *et al.*, 2001 and 2003; Gardner *et al.*, 2004; Boon *et al.*, 2004). Therefore, it would be important to solve these technical difficulties to enable generation of robust data using those constructs.

To summarise this section of work, the finding that cytoplasmic β-catenin is degraded in response to the panel of NSAIDs used here is in keeping with a previous study (Thompson *et al.*, 2000) which reported a reduction in levels of cytoplasmic β-catenin in response to sulindac sulfone and suggests that upstream kinases, for example GSK-β or IKK, could be important in this process. Significantly, the data presents novel observations that β-catenin localises to the nucleolus in response to aspirin, sulindac, sulindac sulfone and indomethacin treatment. The favoured model is that NSAIDs induce degradation of cytoplasmic β-catenin and nucleolar accumulation of nuclear β-catenin. Furthermore, data discussed earlier provides

evidence that RelA is implicated in the mechanism of nucleolar sequestration of β-catenin in the response to aspirin. NSAID effects on β-catenin signaling, in particular nucleolar localisation of β-catenin, could therefore be important in the apoptotic mechanism of NSAIDs, specifically aspirin.

## 7.4 The Validity of the *In vitro* Culture of Colon Cancer Cell Lines as a Model for Chemoprevention

The studies described in this thesis predominantly used the SW480 colon cancer cell line as a model for colorectal cancer. Although this has been shown to be a good model for colorectal cancer (Hewitt *et al.*, 2000), it would be important in the future to make use of other colorectal cancer cell lines, for example HT-29 or HCT-116, and non-colorectal cancer cell lines, to examine the cell-type and tissue specificity of the observed effects with the non-aspirin NSAIDs used in this study. It should be noted that the host laboratory previously demonstrated that aspirin induces dose-dependent degradation of IκB, nuclear translocation of RelA and apoptosis in several colorectal cancer cell lines, but not in the breast, ovarian or endometrial cell lines which were studied, demonstrating that aspirin-effects on the NF-κB pathway are specific to colorectal cancer cell lines (Din *et al.*, 2004). It would also be of interest to make use of an *in vivo* animal model of colorectal cancer, such as the xenograft model, or to examine tissue from human colorectal cancer patients being treated with NSAIDs, in order to see if any of the observed effects of NSAIDs *in vitro* can be replicated *in vivo*. It is worth mentioning that recent data from the host

laboratory has established that the *in vitro* effects of aspirin on the NF-κB pathway can be replicated *in vivo* (Reid *et al.*, 2004). Specifically, using two different animal models (*Min* mice and nude mice xenografted with HT29 colorectal cancer cells), it was discovered that aspirin administration (40-400 mg/kg) causes a dose-dependent decrease in cytoplasmic IκBα levels in tumour protein extracts, an event that is preceded by phosphorylation of the protein and that corresponds with an increase in nuclear RelA levels. Moreover, an increase in apoptosis was observed after aspirin treatment, as determined by caspase-3 cleavage. Preliminary data from the host laboratory also indicates that similar effects of aspirin to those outlined for the animal models are seen in human patient tumour samples.

Another point concerning the use of cell lines as a model for chemoprevention of colorectal cancer is the concentrations of NSAIDs used. In this and previous studies, the anti-tumour effects of NSAIDs are, in general, observed using only relatively high concentrations of these agents. However, previous studies from the host laboratory have demonstrated activation of the NF-κB pathway and cell death at concentrations of aspirin as low as 0.5 mM, a dose that was comparable to salicylate levels measured in the plasma of human subjects (0.5 mM) (Stark *et al.*, 2001; Stark and Dunlop, 2005). Additionally, very recent data from studies of animal models of colorectal cancer conducted in the host laboratory found salicylate levels of 1 mM in mice treated with aspirin (400 mg/kg). Moreover, the lower concentration of aspirin (3 mM) used in this study is relevant to pharmacological salicylate levels used in clinical practice (1-3 mM) (Insel, 1996). In particular, plasma salicylate levels of 1-3 mM are reached in the treatment of patients with rheumatoid arthritis (Pachman *et al.*, 1979). As far as the non-aspirin NSAIDs are

concerned, peak plasma levels of 5.5 μM sulindac (Porter, 1984), 23-30 μM sulindac sulfone (van Stolk *et al.*, 2000) and 1.7 μM indomethacin (Neander *et al.*, 1992) have previously been reported. It would therefore be very important to demonstrate activation of NF-κB and cell death at lower, physiologically relevant dosages of these NSAIDs in future studies. However, it should be emphasized that the concentrations of NSAIDs used here are comparable to those published in other studies (Piazza *et al.*, 1995; Chan *et al.*, 1998; Qiao *et al.*, 1998a; Smith *et al.*, 2000).

A final point concerning the use of NSAIDs, particularly at high doses, in cell culture models of colorectal cancer is the possibility that general toxicity could be responsible for any observed effects of these agents. However, in this study, it was shown that the effects of NSAIDs on NF-κB signaling and apoptosis can be blocked by using the RelA(Δ27-30) dominant negative construct, by using the c-Src inhibitor, PP2, and in cells stably transfected with a kinase dead form of c-Src. Furthermore, previous work in the host laboratory established that the effects of aspirin-induced nucleolar localisation of RelA could be blocked by PDTC (an inhibitor of IκB phosphorylation) or by overexpressing a super-repressor form of IκBα which is resistant to phosphorylation on serines 32 and 36 (Sark *et al.*, 2001; Stark and Dunlop, 2005). Collectively, these data indicate that the effects of NSAIDs on NF-κB signaling and apoptosis are unlikely to be due to non-specific toxic effects.

It must be emphasized that chemoprevention with NSAIDs requires a long time period and so it is difficult to establish suitable models to test in laboratory conditions. Collectively, the data outlined above indicates that the use of cell lines as a model for chemoprevention of colorectal cancer has limitations but also has certain benefits. Specifically, cell lines may be useful to test the anti-tumour activity of

potential chemopreventative agents whose development has been guided by studies that have elucidated the causal pathway involved in NSAID induced anti-tumour effects.

## 7.5 Conclusion

Taken as a whole, the work presented has explored the mechanism whereby NSAIDs induce apoptosis of colorectal cancer cells. A proposed model is summarised in Figure 7.1. The data provides evidence that the NSAIDs, aspirin, sulindac, sulindac sulfone and indomethacin activate NF-κB. The end points of NF-κB activation, specifically nucleolar sequestration of RelA and repression of NF-κB-driven transcriptional activity, were found to be common to all the NSAIDs studied. The favoured hypothesis is that nuclear translocation of NF-κB complexes induced by NSAIDs results in recruitment and transportation of basal NF-κB complexes to the nucleolus, an event that requires a co-factor(s). Once in the nucleolus, NF-κB complexes are sequestered away from their target promoters, leading to a decrease in transcription of anti-apoptotic genes and ultimately, apoptosis. Although activation of the NF-κB pathway was found to be common to NSAIDs, the upstream mechanism differed between aspirin and the non-aspirin NSAIDs studied. Whereas aspirin induces phosphorylation and degradation of IκBα, sulindac, sulindac sulfone and indomethacin activate NF-κB by a mechanism that is independent of IκB degradation and is dependent on c-Src.

In addition to NF-κB, NSAIDs also activate β-catenin. It is proposed that cytoplasmic β-catenin is subject to ubiquitin-mediated proteolysis after aspirin, sulindac sulfone and indomethacin treatment, whereas nuclear β-catenin is sequestered in the nucleolus in response to these agents. Moreover, it is speculated that RelA is required for sequestration of β-catenin in the nucleolus. Cross-regulation of the NF-κB and β-catenin pathways might represent an important regulatory mechanism in response to NSAIDs and the nucleolus could be a key site for such cross-talk. Other potential points of interaction of the NF-κB and Wnt/β-catenin pathways after NSAID treatment may involve IKK, GSK-3β, β-TrCP and c-Src. Post-translational modifications, including acetylation and ubiquitination, of RelA and/or β-catenin are predicted to play a significant role in sequestration of these factors in the nucleolus and may mediate binding of co-factors required for this process.

There is much scope for further research to test this model. However, the findings presented here, in combination with published work from the host laboratory, has significantly progressed understanding of the mechanisms whereby NSAIDs induce apoptosis in colorectal cancer cells. Despite advances in surgery, chemotherapy, radiotherapy and screening procedures, there is still a high mortality associated with colorectal cancer. Therefore, it is of vital importance to understand the mechanisms whereby NSAIDs exert their protective effects against colorectal cancer as this could allow the rational development of novel therapeutic agents. Specifically, the results of this study suggest that the design of small molecules that target RelA and/or β-catenin to the nucleolus could be very important. It should also be emphasized that any new drugs which are developed for the prevention of colon

cancer must not only have efficacy but be safe. The withdrawal of the selective COX-2 inhibitor, rofecoxib, due to increased cardiovascular risk highlights this need.

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