5 EXPERIMENTAL CONFIRMATION AND VALIDATION OF ASSOCIATION BETWEEN PHENOTHIAZINE ACTIVITY AND BRAF MUTATION

5.1 Introduction

In the previous chapter I identified a statistically significant correlation between the activities of phenothiazine compounds and the presence of a BRAF mutation in the NCI-60 cell lines. This chapter reports on the subsequent confirmation of phenothiazine anti-proliferative activity in BRAF mutant NCI-60 cell lines, and validation of the relationship in an independent set of melanoma and colorectal cancer cell lines.

5.2 Results

5.2.1 Confirmation of the anti proliferative activity of phenothiazine derivative compounds in the NCI-60 cell lines

I used the MTS *in vitro* proliferation assay to confirm the demonstrated increased sensitivity of BRAF mutant cell lines to two phenothiazine compounds. It was not, however, for practical reasons feasible to replicate the results in the complete NCI-60 cell line set. I therefore selected a subset of those cells. Five V600E BRAF mutant cell lines were selected from the panel of melanoma cell lines with one line from the colorectal panel. I also selected the one G464V BRAF mutant cell line with a coincident RAS mutation. The BRAF wild type cell lines were selected to include the single melanoma cell line in the NCI-60 without a BRAF mutation and one RAS mutant colorectal cancer cell line. The other BRAF wild type cell lines

consisted of a renal, breast, and prostate cancer cell line, each selected as representatives of another tissue type.

I assessed the anti-proliferative activity of two piperazine phenothiazine compounds (NSC 46061, NSC 17474) in those eleven cell lines of the NCI-60. Serially diluted concentrations of the phenothiazine compounds and an untreated control were tested in triplicate. The cell lines were treated with phenothiazine compounds for 48 hours.

The sigmoidal dose response curves represent each cell line's response profile to phenothiazine treatment. The sigmoidal curves are plotted with the x-axis labeled with the $\log_{10}$ of the drug concentration. The y-axis is labeled with the measured absorbance at 490nm of reduced formazan product. The production of formazan is proportional to the number of living cells. Therefore the absorbance measures are an indication of cell viability after 48 hours of drug treatment. The absorbance measures are normalized to that of control untreated cells, labeled as such to the left of the x-axis. The concentration of drug that induces a response halfway between the baseline and maximum, the EC_{50} value, is unique to each drug-cell line pair. The results at each concentration are presented as the mean of three replicate experiments ± standard error of the mean (S.E.M).

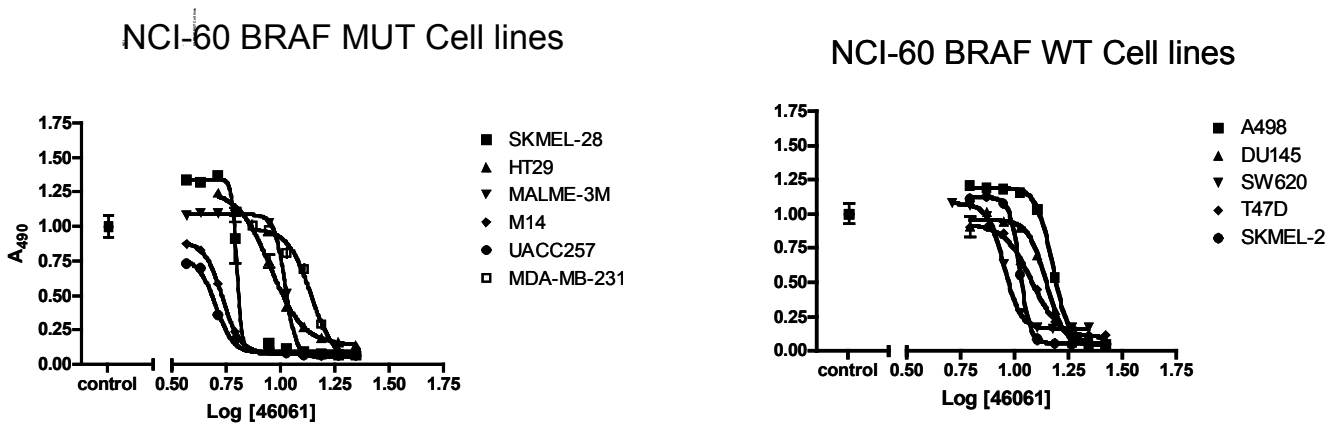

Figure 5-1: Sigmoidal dose response curves of BRAF mutant (MUT) and wild type (WT) cell lines of the NCI-60 following 48 hours of treatment with piperazine phenothiazine compound NSC 46061.

NSC46061 best-fit values calculated from sigmoidal dose-response curves				
Cell line	Tissue type	BRAF status	LOG EC_{50}(μM)±SEM	EC_{50}(μM)
SKMEL-28	Melanoma	Mutant V600E	0.79±0.08	6.29
HT29	Colorectal	Mutant V600E	0.95±0.01	9.01
MALME-3M	Melanoma	Mutant V600E	1.02±0.00	10.52
M14	Melanoma	Mutant V600E	0.73±0.00	5.40
UACC-257	Melanoma	Mutant V600E	0.70±0.00	5.04
MDA-MB-231	Breast	Mutant G464V	1.14±0.00	13.81
A498	Renal	Wild type	1.17±0.00	15.05
DU145	Prostate	Wild type	1.14±0.00	14.03
SW620	Colorectal	Wild type	0.95±0.00	8.95
T47D	Breast	Wild type	1.08±0.00	12.01
SKMEL-2	Melanoma	Wild type	1.02±0.00	10.60

Table 5-1: Best-fit values calculated from the sigmoidal dose response curves of NCI-60 cell lines following 48 hours of treatment with NSC 46061.

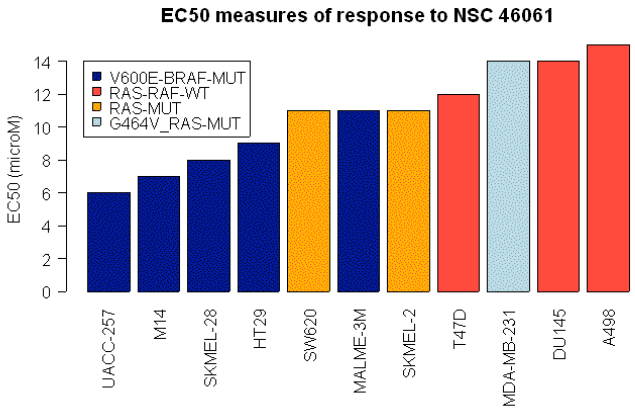

Figure 5-2. EC_{50} values of NCI-60 cell lines treated with piperazine phenothiazine compound NSC 46061. Cell lines are colored according to mutation in BRAF and RAS.

I observe that the V600E BRAF mutant cell lines treated with NSC 46061 have EC_{50} values ranging from 6 μM to 11 μM and the EC_{50} values of V600E BRAF wild type cell lines ranges from 11 μM to 15 μM. The G464V BRAF mutant cell line, in contrast with the results obtained in the initial DTP screen, has an EC_{50} value of 14 μM, similar to that of BRAF wild type cell lines (Figures 5-1 and 5-2, Table 5-1).

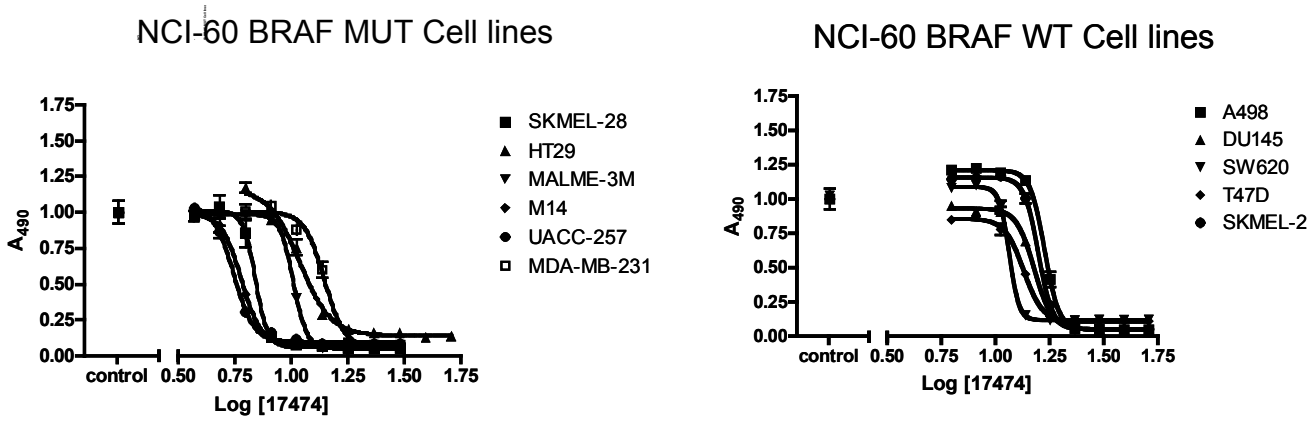

Figure 5-3. Sigmoidal dose response curves of BRAF mutant (MUT) and wild type (WT) cell lines of the NCI-60 following 48 hours of treatment with piperazine phenothiazine compound NSC 17474.

NSC17474 best-fit values calculated from sigmoidal dose-response curves				
Cell line	Tissue type	BRAF Status	LOG EC_{50}(μM)±SEM	EC_{50}(μM)
SKMEL-28	Melanoma	Mutant V600E	0.84±0.00	6.94
HT29	Colorectal	Mutant V600E	1.03±0.01	10.90
MALME-3M	Melanoma	Mutant V600E	1.00±0.00	10.21
M14	Melanoma	Mutant V600E	0.77±0.00	6.01
UACC-257	Melanoma	Mutant V600E	0.74±0.00	5.55
MDA-MB-231	Breast	Mutant G464V	1.14±0.00	14.03
A498	Renal	Wild type	1.22±0.00	16.88
DU145	Prostate	Wild type	1.18±0.00	15.12
SW620	Colorectal	Wild type	1.06±0.00	11.58
T47D	Breast	Wild type	1.13±0.00	13.50
SKMEL-2	Melanoma	Wild type	1.19±0.00	15.81

Table 5-2: Best-fit values calculated from the sigmoidal dose response curves of NCI-60 cell lines following 48 hours of treatment with NSC 17474.

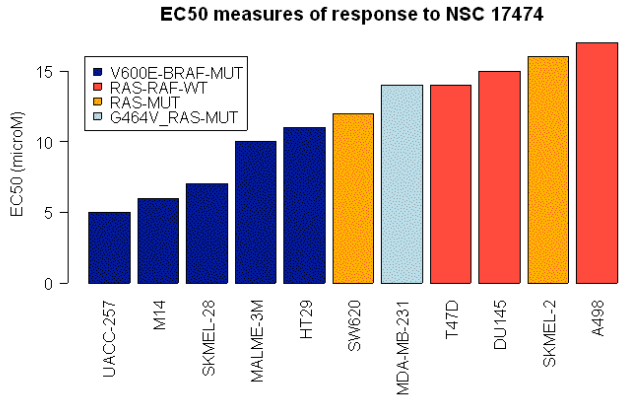

Figure 5-4. EC_{50} values of NCI-60 cell lines treated with piperazine phenothiazine compound NSC 17474. Cell lines are colored according to mutation in BRAF and RAS.

The V600E BRAF mutant cell lines treated with NSC 17474 have EC_{50} values ranging from 5 μM to 11 μM and that of the BRAF wild type cell lines ranges from 10 μM to 17 μM. The G464V BRAF mutant cell line has an EC_{50} value of 14 μM (Figures 5-3 and 5-4, Table 5-2), similar to those of the BRAF wild type cell lines and consistent with the initial results obtained in the DTP screen.

Overall, the general trend of phenothiazine activity showed that V600E BRAF mutant cell lines are more sensitive than are the G464V BRAF mutant, RAS mutant, and RAS/RAF wild type lines. Piperazine phenothiazines demonstrated activity at EC_{50} values ranging between 5 μM and 18 μM, with EC_{50} values ranging from 5 μM to 11 μM in V600E BRAF mutant melanomas.

The EC_{50} values were, thus, two-fold higher in V600E BRAF wild type cell lines, including the one NRAS (Q61R) mutant melanoma cell line, SKMEL-2. Excluding the EC_{50} value of the G464V BRAF mutant cell line, the differential response to NSC 46061 for V600E BRAF mutants and wild type cell lines was statistically significant (p-value = 0.020, Wilcoxon p-value < 0.05). The differential response to NSC 17474 for V600E BRAF mutant and wild type cell lines was also statistically significant (p-value = 0.007, Wilcoxon p-value < 0.05).

5.2.2 Validation of differential sensitivity of V600E BRAF mutant and RAS mutant melanoma

Following the confirmation of differential phenothiazine activity in the NCI-60 cell lines based on V600E BRAF mutation, I sought to replicate the finding. First I examined a larger and independent set of melanoma cell lines. I obtained thirteen additional melanoma lines with various *BRAF* and *RAS* mutations and performed MTS assays following 48 hours of treatment with piperazine phenothiazine compound NSC 17474 (Figure 5-9). NSC 17474 was chosen because it showed a bigger differential EC_{50} measures than NSC 46061 between V600E BRAF mutant melanomas and the BRAF wild type cell lines.

I observed that the V600E BRAF mutant melanoma cell lines had EC_{50} measures ranging from 5 μM to 8 μM. Interestingly the melanoma cell lines with V600D and V600K BRAF mutation had EC_{50} values of 9 μM and 5 μM, respectively similar to that of the V600E BRAF mutants. The RAS mutant

melanoma cell lines had EC_{50} values ranging from 10 μM to 18 μM. Within the group of RAS mutant melanoma cell lines there were two with interesting mutation combinations. The first, MEL-JUSO, has both an NRAS (Q61L) and HRAS (G13D) mutation, and the EC_{50} value I obtained was 10 μM. The second melanoma cell line of interest is HMVII, which has BRAF (G469V) and an NRAS (Q61K) mutations and an EC_{50} value of 14 μM. The RAS/RAF wild type melanoma cell lines had EC_{50} values of 14 μM and 16 μM. Those EC_{50} values are similar to that of the RAS mutant melanoma cell lines.

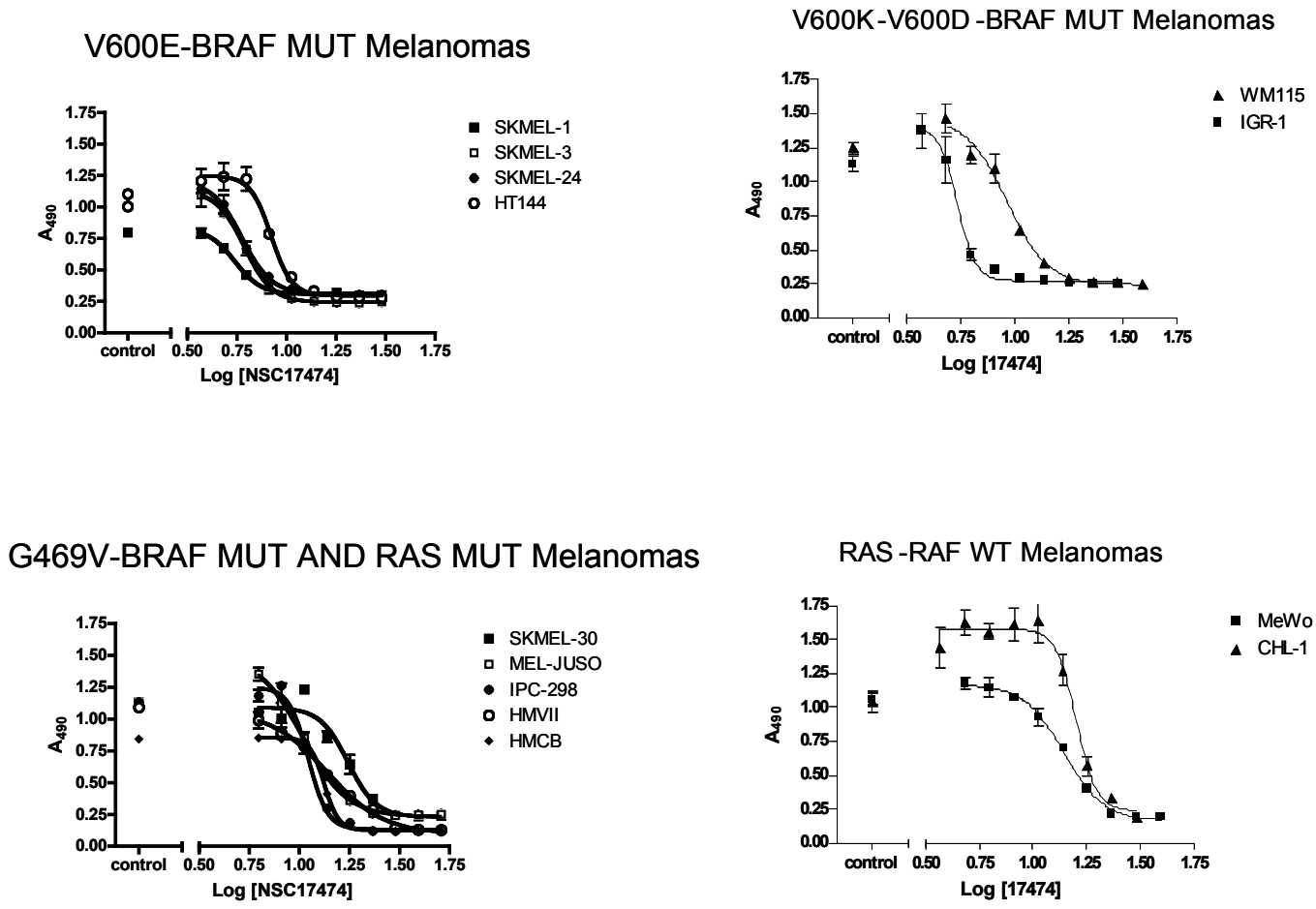

Figure 5-9. Sigmoidal dose response curves of V600E BRAF mutant, V600K and V600D BRAF mutant, RAS mutant, and RAS/RAF wild type melanoma

cell lines following 48 hours of treatment with piperazine phenothiazine compound NSC 17474.

NSC17474 best-fit values calculated from sigmoidal dose-response curves				
Cell line	Tissue type	BRAF status	LOG EC_{50}(μM)±SEM	EC_{50}(μM)
SKMEL-1	Melanoma	Mutant V600E	0.73±0.01	5.47
SKMEL-3	Melanoma	Mutant V600E	0.78±0.01	6.13
SKMEL-24	Melanoma	Mutant V600E	0.77±0.01	5.94
HT144	Melanoma	Mutant V600E	0.92±0.01	8.36
IGR-1	Melanoma	Mutant V600D	0.73±0.01	5.43
WM115	Melanoma	Mutant V600K	0.96±0.01	9.23
SKMEL-30	Melanoma	Wild type	1.24±0.02	17.52
MEL-JUSO	Melanoma	Wild type	1.01±0.03	10.40
IPC-298	Melanoma	Wild type	1.04±0.00	11.09
HMVII	Melanoma	Mutant G469V	1.13±0.02	13.79
HMCB	Melanoma	Wild type	1.12±0.00	13.33
MeWo	Melanoma	Wild type	1.14±0.01	13.94
CHL-1	Melanoma	Wild type	1.20±0.01	15.88

Table 5-3: Best-fit values calculated from the sigmoidal dose response curves of an independent set of melanoma cell lines following 48 hours of treatment with NSC 17474.

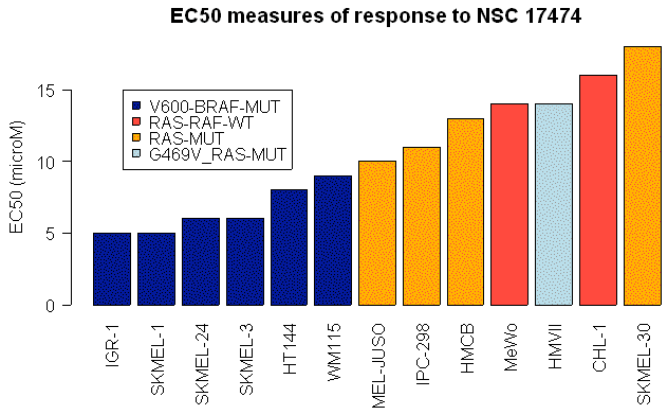

Figure 5-10. EC_{50} values of NSC 17474 in an independent set of melanoma cell lines. Cell lines are colored according to mutation in BRAF and RAS.

Based on the EC_{50} values of an independent set of thirteen melanoma cell lines, I found that BRAF mutation at codon 600 is predictive of an increase in sensitivity to inhibition by NSC 17474 when compared with all other genotypes studied (Figures 5-10 and 5-11, Table 5-3). The results also demonstrate that the presence of RAS mutation and/or non-codon 600 BRAF mutation is associated with decreased sensitivity to inhibition by NSC 17474 compared to codon 600 BRAF mutants (p-value = 0.007, Wilcoxon p-value < 0.05). Similarly, absence of both RAS and BRAF mutation in melanoma cell lines is associated with decreased sensitivity to inhibition by NSC 17474 compared to V600E BRAF mutants.

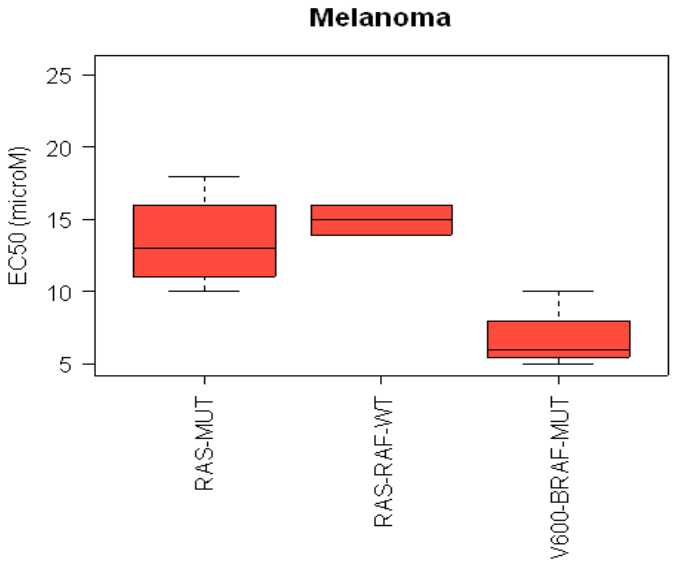

Figure 5-11. Box and whisker plots of the EC_{50} values of phenothiazine-NSC 17474- tested in RAS mutant (MUT), RAS/RAF wild type (WT), and BRAF mutant (MUT) melanoma cell lines.

5.2.3 Validation of differential sensitivity of V600E BRAF mutant and RAS mutant colorectal cancer cell lines

After validating the predicted differential sensitivity between V600E BRAF mutant and RAS mutant melanomas to NSC 17474, I hypothesized that the phenomenon may extend to other tissue types. I had previously observed suggestions of a similar pattern of preferential sensitivity to phenothiazines in V600E BRAF mutant colorectal cancer cell lines of the NCI-60. However, there are only two V600E BRAF mutant colorectal cancer cell lines in the NCI-60. Therefore, more V600E BRAF mutant colon lines would need to be tested

to make a confident assertion of an influence of mutation on response to phenothiazines. Therefore, I acquired an independent set of eleven colorectal cancer lines with various BRAF and RAS mutations. Similar to the treatment of melanomas, I performed MTS assays on this larger set of colorectal cancer cell lines following 48 hours of treatment with NSC 17474 (Figure 5-12).

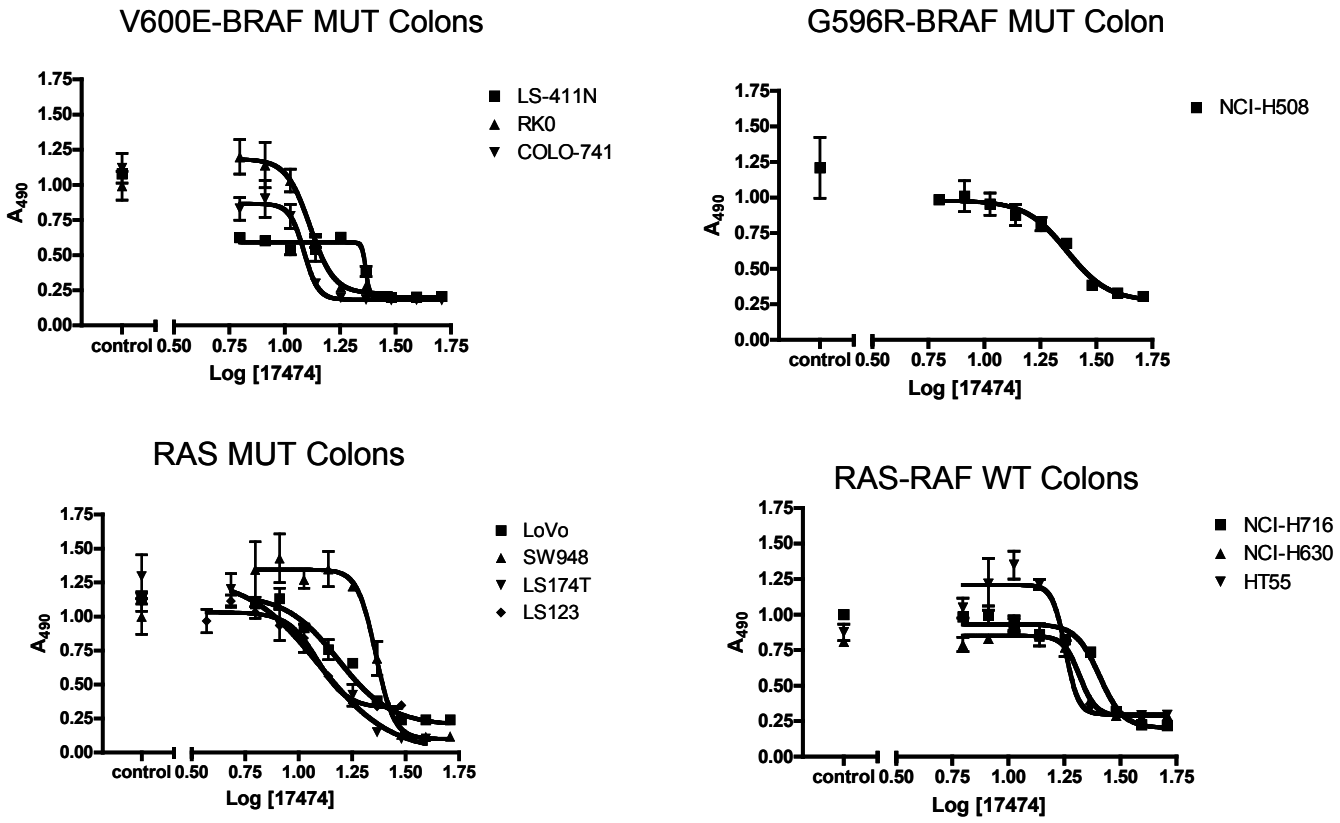

Figure 5-12. Sigmoidal dose response curves of V600E BRAF mutant, G569R BRAF mutant, RAS mutant, and RAS/RAF wild type colorectal cancer cell lines following 48 hours of treatment with piperazine phenothiazine compound NSC 17474.

NSC17474 best-fit values calculated from sigmoidal dose-response curves				
Cell line	Tissue type	BRAF status	LOG EC_{50}(μM)±SEM	EC_{50}(μM)
LS-411N	Colorectal	Mutant V600E	1.36±2.66	23.23
RKO	Colorectal	Mutant V600E	1.11±0.02	13.02
COLO-741	Colorectal	Mutant V600E	1.08±0.01	12.22
NCI-H508	Colorectal	Mutant G596R	1.36±0.03	23.37
LoVo	Colorectal	Wild type	1.18±0.02	15.34
SW948	Colorectal	Wild type	1.36±0.01	22.94
LS174T	Colorectal	Wild type	1.09±0.07	12.46
LS123	Colorectal	Wild type	1.08±0.02	12.23
NCI-H716	Colorectal	Wild type	1.40±0.01	25.53
NCI-H630	Colorectal	Wild type	1.31±0.01	20.81
HT55	Colorectal	Wild type	1.25±0.01	17.98

Table 5-4: Best-fit values calculated from the sigmoidal dose response curves of an independent set of colorectal cell lines following 48 hours of treatment with NSC 17474.

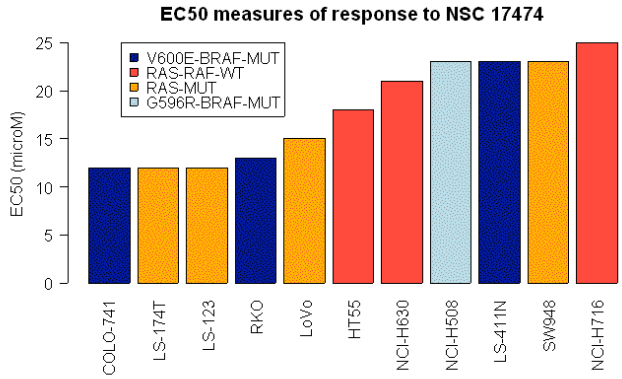

Figure 5-13. EC_{50} values of NSC 17474 in an independent set of colorectal cell lines. Cell lines are colored according to mutation in BRAF and RAS.

The V600E BRAF mutant colorectal cancer cell lines measured had EC_{50} values ranging from 12 μM to 23 μM. However, the calculated EC_{50} value of 23 μM for colorectal cell line LS-411N cannot be considered accurate

because of a large SEM. The G569R BRAF mutant colorectal cell line has an EC_{50} value of 23 μM. The RAS mutant colorectal cancer cell lines have EC_{50} values ranging from 12 μM to 23 μM. The RAS/RAF wild type colorectal cancer cell lines have EC_{50} values ranging from 18 μM to 25 μM.

Based on the EC_{50} values I obtained for an independent set of eleven colorectal lines, the pattern of response to NSC 17474 is different from that observed in the melanoma cell lines (Table 5-4, Figure 5-13). I found that BRAF and RAS mutant colorectal cancer cell lines had similar patterns of response to NSC 17474 (p-value = 1.0, Wilcoxon p-value < 0.05). Although the RAS/RAF wild type colorectal cancer cell lines had approximately two-fold higher EC_{50} values than the BRAF mutant and RAS mutant colorectal lines, there is considerable overlap between the groups (Figure 5-14).

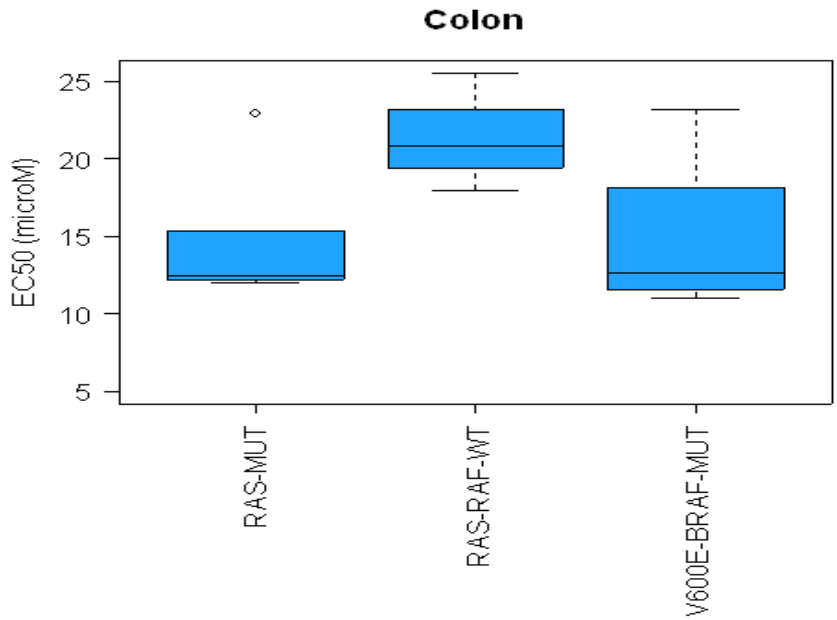

Figure 5-14. Box and whisker plots of the EC_{50} values of phenothiazine-NSC 17474- tested in RAS mutant (MUT), RAS/RAF wild type (WT), and BRAF mutant (MUT) colorectal cell lines.

5.3 Discussion

Overall, I confirmed the statistical association between increased anti-proliferative activity of phenothiazines and BRAF mutation in melanoma. Interestingly, I observed that the increased activity of phenothiazine compounds in BRAF mutant cell lines of the NCI-60 seemed to be based on the presence of V600E BRAF mutation and did not extend to the G464V BRAF mutation. However, I did not have enough cell lines of differing BRAF and RAS mutation status to make that assertion with confidence. In a larger and independent set of melanoma cell lines, I demonstrated that the

increased phenothiazine activity in melanomas is most apparent with codon 600 BRAF mutant melanomas. I found that non- codon 600 BRAF mutant, RAS mutant and RAS/RAF wild type melanomas exhibited less sensitivity to piperazine phenothiazine than did the codon 600 BRAF mutants.

The kinase activities of different codon 600 BRAF mutants are similar to one another (Wan et al. 2004). Therefore, it is likely that all codon 600 BRAF mutants would respond similarly to drug inhibition, as I have demonstrated with phenothiazines. However, I had only two additional codon 600, non-V600E, BRAF mutants and would need larger numbers of such mutants to confirm this finding. The caveat is that codon 600 BRAF mutants other than V600E, are rare. It is also known that mutations of the glycine-rich loop (G loop) of BRAF such as the G464V mutant and G469V mutant do not confer the same kinase activation to the BRAF protein as the codon 600 mutants. The G464V BRAF mutation is known to increase the kinase activity of BRAF modestly compared to codon 600 BRAF mutants (Davies et al. 2002). Whereas the codon 600 BRAF mutation confers constitutive kinase activity on the BRAF protein, the G loop BRAF mutants primarily disrupt the G loop and kinase domain interaction, destabilizing the inactive BRAF conformation and stimulating BRAF activity (Garnett and Marais 2004). The kinase activity of the G469V mutant has not been tested. However a different amino acid substitution at codon 469 (G469A) has been shown to have high basal kinase activity comparable with V600E BRAF mutants (Davies et al. 2002). The drug response data show that the G469V BRAF mutant

melanoma cell line was not as sensitive as the codon 600 BRAF mutants to inhibition by phenothiazines.

There appeared to be a similar trend, as in the melanomas, of increased phenothiazine activity in the two V600E BRAF mutant colorectal cancer cell lines of the NCI-60 compared with the RAS mutant colorectal cancer cell lines. In a larger and independent set of eleven colorectal cancer cell lines, I observed that the presence of V600E BRAF mutation was not associated with an increased sensitivity to phenothiazine. There was also no difference in the EC_{50} values between V600E BRAF mutant, G596R BRAF mutant and RAS mutant colorectal cancers. In fact, the EC_{50} values of the BRAF mutants, RAS mutants and RAS/RAF wild type colorectal cancer cell lines overlapped.

That finding may mirror the different biological contexts of BRAF mutation in melanomas and colorectal cancers. The V600E BRAF mutation is the most common BRAF mutation in both melanomas and colorectal cancers. BRAF mutation is an initiating event in melanoma. BRAF mutant and RAS mutant melanomas activate ERK differently. BRAF mutants, especially the V600E mutants, directly activate MEK. However, RAS mutant melanomas activate wild type CRAF that then activates MEK (Wan et al. 2004). In colorectal tumor development, BRAF mutation occurs in the adenoma-to-carcinoma sequence, identical to the stage at which KRAS mutations occur (Yuen et al. 2002). Therefore, it has been proposed that BRAF and KRAS mutation in colorectal cancers have similar phenotypic patterns (Yuen et al.

2002). The V600E BRAF mutation in colorectal cancers also leads to direct phosphorylation of MEK. However, the G596R BRAF mutation found in colorectal cancer causes a loss of kinase activity to the BRAF protein. Interestingly, however, the G596R mutant is still capable of stimulating ERK via activation of wild type CRAF (Wan et al. 2004).

Biologically, V600E BRAF and RAS mutations in melanomas are quite distinct from one another. In colorectal cancers, V600E BRAF and RAS mutation seem to confer similar phenotypes. Perhaps this biological difference is reflected in the pharmacological response of the melanomas and colorectal cancers to phenothiazines.

Despite the relatively small numbers of melanoma cell lines I have tested, the results support an association between the presence of V600E BRAF mutation and increased phenothiazine activity. Importantly, I have demonstrated that the phenothiazine activity is not solely due to melanoma status, but is due to the presence of the V600E BRAF mutation within a melanoma context. I also observed that the size of effect after treatment with phenothiazines between the BRAF mutant and wild type cell lines in the initial NCI-60 screen was approximately six-fold. However, in my confirmation studies the size of effect was approximately two-fold. That difference can be due to several reasons. Firstly, it could be due to the difference between the *in vitro* assays used to measure the effect. The DTP NCI-60 screen used a sulforhodamine B (SRB) assay, which measures protein content as a treatment endpoint. I used the MTS assay, which measures the reduction of

a tetrazolium salt to formazan product as an indicator of the number of metabolically active or viable cells relative to control after drug treatment. The two assays are different and hence can produce different magnitudes of effect. Secondly, it could be due to the difference in the experimental setup and analysis. The DTP screen used five standard ten-fold serial dilutions of phenothiazine compounds to assess the GI_{50} values. I employed a much more meticulous analysis by using 1.2-fold and 1.3-fold serial dilutions of phenothiazine compounds to assess the EC_{50} values and performed more experimental repeats than was done in the DTP screen. However, importantly, the trend of effect is consistent between the sets of results.

I have validated the trend of phenothiazine activity and presence of V600E BRAF mutation in a set of thirteen melanomas and eleven colorectal cancers. It would also be interesting to investigate whether this differential effect of phenothiazines also occurs in other tissue types with similar BRAF and RAS mutations such as thyroid cancers.

Phenothiazines, a class of psychotropic drugs, have been used to treat schizophrenia and other psychiatric illnesses due to their antagonist activity on dopamine (D2) receptors. In recent years some psychotropic agents have been described as possessing anti-proliferative activity (Nordenberg et al. 1999). Also recently, epidemiological studies have shown that schizophrenic patients have decreased risk of certain cancer types including melanoma (Mortensen 1994, Cohen et al. 2002, Dalton et al. 2005, Grinshpoon et al. 2005). Use of neuroleptic medications has been shown to correlate with a

suggestive decrease in rectal, colon and prostate cancers (Dalton et al. 2006). However, it is not clear if use of neuroleptics or schizophrenia or both is causally associated with decreased risk of melanoma. Numerous studies have demonstrated, using *in vitro* and *in vivo* methods, that phenothiazines cause a dose-dependent decrease in cell viability and induce apoptosis via an increase in caspase-3 activity and in fragmentation of DNA in cancer cell lines of diverse origin: IRSC-10M small cell lung cancer cells (Zhu et al. 1991), HL-60 leukemic cells (Schleuning et al. 1993) SH-SY5Y neuroblastoma (Gil-Ad et al. 2004), and B16 mouse melanoma cells (Gil-Ad et al. 2006).

Phenothiazines are also often prescribed as anti-emetics during cancer chemotherapy. Interestingly, some phenothiazine derivative compounds have been shown to enhance the cytotoxic effect of cancer chemotherapeutic drugs. Trifluoperazine, a piperazine derivative phenothiazine compound, has been shown to potentiate the DNA damaging effect of cisplatin in a non-small cell lung cancer cell (Eriksson et al. 2001). A high throughput screen of thousands of combinations of existing drugs revealed that the combination of chlorpromazine, an aliphatic derivative phenothiazine, and pentamidine, an anti-infective drug, has a synergistic effect in killing A549 lung carcinoma cell lines (Borisy et al. 2003). The demonstrated synergy of phenothiazine compounds and cancer chemotherapeutic agents in *in vitro* studies has been patented for possible future clinical application (Borisy et al. 2003).

As well, some phenothiazine derivatives have greater activity in neoplasms than in normal tissue. Human neuroblastoma and rat glioma cells

have been shown to be more sensitive to thioridazine, a piperidine derivative phenothiazine, than primary whole-brain culture (Gil-Ad et al. 2004). Phenothiazine compounds, trifluoperazine, thioridazine, and chlorpromazine, at concentrations up to 20 μM, expressed antiproliferative activity and induced apoptosis in leukemic cells without any influence on the viability of normal lymphocytes (Zhelev et al. 2004).

Several different mechanisms have been proposed to explain the antiproliferative activity of phenothiazines: inhibition of protein kinase C activity (Zhu et al. 1991), reversal of multidrug resistance (MDR) (Hait et al. 1992, Nordenberg et al. 1999), calcium channel-blocking and calmodulin antagonistic activity (Barancik et al. 1994), and inhibition of DNA-dependent protein kinase C (DNA-PK) (Eriksson et al. 2001). However, the underlying mechanism is not yet known.

To our knowledge this is the first report of an increased sensitivity of melanoma cell lines to inhibition by piperazine phenothiazines. The differential response of melanomas may be evidence of the underlying antiproliferative mechanism of action of piperazine phenothiazine compounds. It would be of interest to understand the mechanism of the increased efficacy of piperazine phenothiazines in codon 600 BRAF mutant melanomas. It may also be of interest to dissect the myriad cellular pathways that may be affected by phenothiazines. Based on a review of current literature surrounding the elucidation of molecular effects of phenothiazine compounds

and the biological regulation of RAS- RAF- MEK- ERK-MAPK pathway, I have developed a working theory of the cellular effects of phenothiazines.

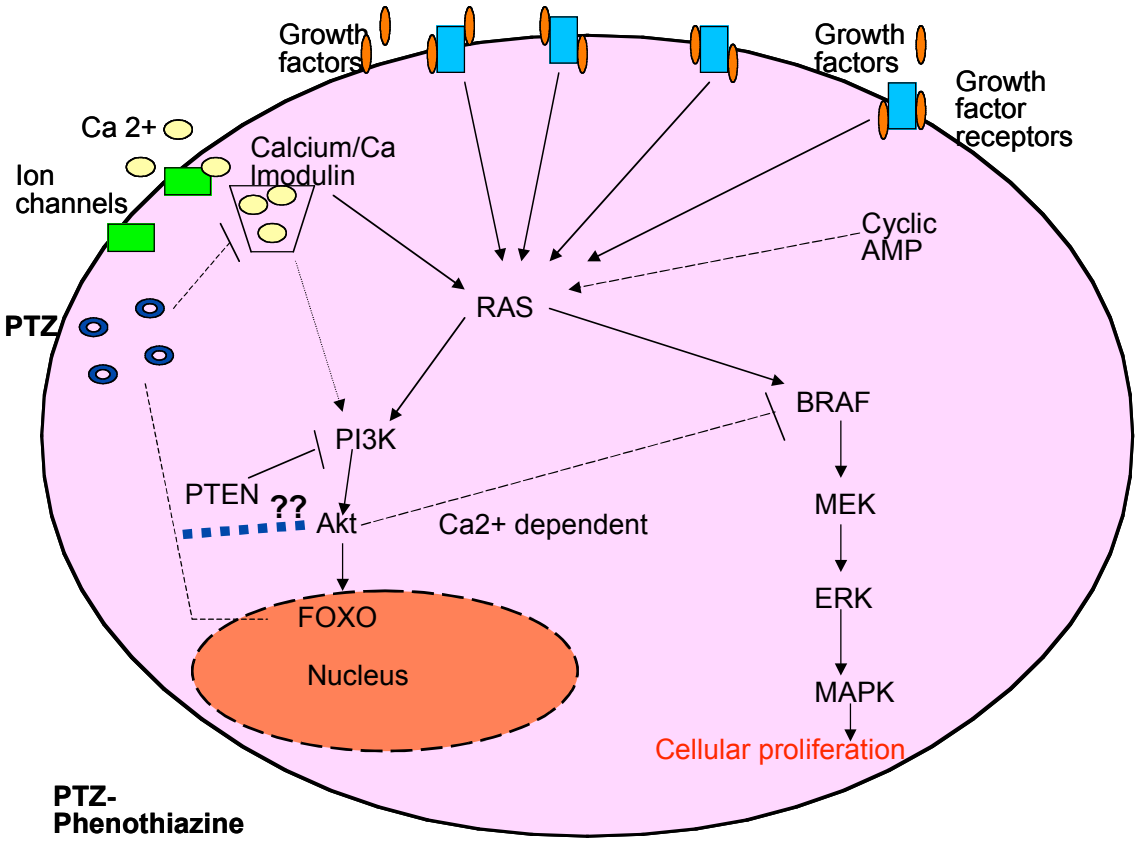

Figure 5-15. A schematic of a normal human cell and RAS regulated pathways involved in cellular proliferation and growth. Phenothiazines' mechanism of action is also diagrammed based on interpretation of the current knowledge of the drug's action inside the cell. In this schematic, piperazine phenothiazine NSC 17474 within the cell inhibits calmodulin, thereby releasing intracellular stores of calcium. This increase in cytoplasmic calcium triggers activation of the AKT pathway, resulting in two effects. The first is inhibition of relocalization of FOXO1a transcription factor to the nucleus

in PTEN null cancer cells (Kau et al. 2003). The second is an increased regulation of BRAF by AKT (Yamaguchi et al. 2004). This second effect, however, is also based on the presence of cyclic AMP stimulation on RAS and BRAF (Yamaguchi et al. 2004).

In our current understanding, AKT negatively regulates BRAF directly, in a calcium dependent manner. Therefore, an increase in intracellular calcium stores due to inhibition of calmodulin can decrease the proliferative activity of BRAF through regulation by AKT (Figure 5-15).

Another possible theory is that phenothiazines directly inhibit BRAF. It has been shown that a piperazine phenothiazine inhibits DNA protein kinase (DNA-PK) a serine/threonine kinase (Eriksson et al. 2001). The inhibitory effect of DNA-PK by the piperazine phenothiazine was achieved at a concentration of 100 μM (Eriksson et al. 2001). However, the antiproliferative activity of phenothiazines is achieved at much lower doses (5μM to 10μM) in V600E BRAF mutants. BRAF and ERK are serine/threonine kinases, and it may be that phenothiazines non-specifically bind to and inhibit the activity of serine threonine kinases. Given that V600E BRAF mutation accompanies *MITF* amplification in the melanoma lines of the NCI-60, it may also be likely that phenothiazines modulate the regulation of proliferation by MITF, sensitizing those melanoma lines to apoptosis (Garraway et al. 2005).

To explore further the mechanism of the increased sensitivity of V600E BRAF mutant melanoma to phenothiazines, it would be important to evaluate the downstream effectors of BRAF, such as MEK and ERK, following phenothiazine treatment. In addition to elucidating the mechanism of the anti proliferative activity of phenothiazines, it would be important to recapitulate the differential sensitivity to phenothiazines in vivo, in mice harboring xenograft tumors. A potential problem is that in vitro drug response is not always predictive of in vivo response. Secondly, the magnitude of the differential response to phenothiazines, as measured by MTS assay, is small and may not be measurable in in vivo experiments. However, experiments in vivo would need to be performed to evaluate the possible therapeutic benefit of phenothiazines for patients diagnosed with melanoma.