5 DISCUSSION

5.1 VALIDATION STUDY

5.1.1 MORPHOLOGICAL CHANGES IN THE RAT MODEL

After establishing a rat model of OAC in which adenocarcinoma developed in response to reflux of proximal small bowel contents following oesophagojejunostomy we characterised the sequence of histological and morphological changes that occurred in the oesophagus following surgery. Our results confirmed that the gross morphological changes observed in the rat oesophagus in this study are similar to those reported in this and other animal models of OAC in response to reflux induced injury (Goldstein et al 1997, Melo et al 1999, Buttar et al 2002). All the operated animals developed severe inflammation of the distal oesophagus with marked squamous hyperplasia and a short segment of BO within 4 weeks of surgery. The Barrett's segment was invariably located immediately proximal to the anastomosis with an area~~intervening bridge~~ of oesophageal squamous epithelium between the Barrett's segment and jejunal mucosa. The length of the Barrett's segment increased with time from surgery. The first OAC was observed 8 weeks following surgery and the incidence of carcinoma increased over time. All carcinomas were well-differentiated mucinous adenocarcinomas and originated from the distal oesophagus above the oesophagojejunal anastomosis. Eighty four percent of carcinomas originated within an island of Barrett's oesophagus.

There are many morphological similarities between the rat model and human disease. In both the rat model and humans the primary site of disease is the distal oesophagus. The histological changes in the rat oesophagus in response to reflux induced injury closely mirror the histological changes reported to occur in human disease. In the early stages of disease these include erosions and ulceration with basal cell hyperplasia and elongation of the dermal papillae. These changes are usually associated with a~~n~~ mixed inflammatory cell infiltrate in the oesophageal mucosa. Subsequent changes in the mucosa include epithelial hyperplasia, columnar metaplasia, and with the appearance of goblet cells within the columnar epithelium, intestinal metaplasia or Barrett's oesophagus (Riddell 2005). Further, OAC in humans is associated with intestinal metaplasia, and usually develops within an island of BO. Clark et al reported that 79% of OAC were associated with BO after histological examination of pathological specimens following oesophagectomy in 48 patients (Clarke et al 1994). In the rat model, BO was associated with OAC, and a similar proportion of cancers (84%) were observed to arise within an island of BO.

In addition, the cumulative risk of developing Barrett's associated OAC in humans increases with the duration of time BO has been present. A similar pattern of disease incidence was seen in the rat, with an increasing incidence of OAC with time from induction of reflux.

However, all the tumours that developed in the rat were mucinous well-differentiated adenocarcinomas. This differs from human disease in which the predominant tumour types are well or moderately differentiated adenocarcinoma. Mucinous adenocarcinoma does occur in humans but is less common.

5.1.2 CpG ISLAND HYPERMETHYLATION

One of the molecular changes associated with Barrett's neoplasia that is currently receiving a great deal of attention is the development of CpG-island methylation, both in the tumour, and as a field effect in the surrounding mucosa. Aberrant hypermethylation of the promoter region of tumour suppressor genes can lead to inactivation of those genes, and may thereby promote carcinogenesis. There are a number of such genes in which CpG hypermethylation is reported to occur during the development of OAC including *ESR-1, p16,* and *HPP1*.

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In the present study we used a novel and extremely sensitive quantitative assay to compare the absolute levels of methylation of defined CpG dinucleotides in the *ESR-1, p16* and *HPP1* promoter region in jejunal, squamous and Barrett's mucosa, as the disease process developed following surgery. Quantitative analysis of CpG island methylation presents technical difficulties that are not always widely appreciated or acknowledged. Of the alternative techniques available for the detection of sequence differences, methylation specific PCR (MSP) is probably the most widely used. However it is essentially a qualitative method which distinguishes the presence or absence of methylated sequences in the starting

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template, down to limits of detection that are very low, but not stated in most reports. As has been emphasised recently by Ogino et al, when assessing the mechanistic significance of CpG island methylation in genes associated with disease processes, it is extremely important to use quantitative techniques (Ogino et al 2006). At present the quantitative assay most commonly used is MethyLight which, like the method described here, is based on the use of real-time PCR reactions. The percentage of methylated cytosines at the locus of interest is then calculated by comparing the normalised result for the sequence of interest to that of a fully methylated reference to provide a "percentage of methylated reference" value (PMR). In practice however MethyLight has been used mainly to classify tumours or pre-malignant tissues into "methylated" or "unmethylated" sub-groups, often with an arbitrary lower cut-off PMR of between 4 and 10% (Eads et al 2001). Consequently there is a paucity of qualitative data in the literature reporting absolute levels of CpG island methylation at any gene locus within a given population of patients with BO or OAC.

Our quantitative analysis of CpG island methylation in the *ESR-1*, *p16*, and *HPP1* promoter regions in the rat model detected methylation occurring throughout the metaplasia – dysplasia – carcinoma sequence at all three gene loci. We detected levels of CpG island methylation in the *ESR-1* promoter region from four weeks after surgery in BO in all operated rats, at levels that would be undetectable by other methods. Low levels of methylation were also present in all rats 16 weeks after surgery, and a statistically significant increase in CpG island methylation of

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the promoter region of *ESR-1* was observed 28 weeks following surgery in both BO and OAC. Low levels of *p16* promotor hypermethylation were also detected in all the rats from 4 weeks after surgery. Again, the level of methylation in the *p16* promoter increased as the disease progressed through the metaplasia-dysplasia-carcinoma sequence, and there were significantly higher levels of methylation of *p16* in OAC at 28 weeks. There was also a progressive and significant increase in CpG hypermethylation of the *HPP1* promoter in the rat oesophagus from 4 weeks after surgery, with the highest levels detected in Barrett's oesophagus at 28 weeks. Significantly higher levels of methylation were also present in OAC at 28 weeks.

CpG island hypermethylation of the promoter region is reported to occur at a high frequency~~ies~~ in both BO and OAC in humans at all three of these gene loci. Hypermethylation of the *ESR-1* promoter may affect up to 86% of patients, with *p16* hypermethylation reported in up to 41% of patients, and *HPP1* methylation seen in up to 71% of patients with OAC (Eads et al 2001, Brock et al 2003, Schulmann et al 2005). Hypermethylation in all 3 of these gene promoter regions is reported to increase in frequency as the disease progresses through the metaplasia – dysplasia – carcinoma sequence, with both the highest frequency of methylation and the highest absolute levels of methylation seen in OAC.

Both the presence of and the pattern of CpG island methylation in the *ESR-1*, *p16*, and *HPP1* promotors in the rat esophagus are similar to those seen in humans. However, the levels of methylation observed in the rat model are lower than those

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described in human disease. In the fields of methylation associated with Barrett's oesophagus described by Eads et al, methylation of the *ESR1* and *p16* promoter regions was in excess of 4% (Eads et al 2001). Currently there is no quantitative data in the English literature relating to methylation of *HPP1* in OAC, although Schulmann et al found that this gene promoter is hypermethylated in both BO and OAC in a high proportion of patients with the disease (Schulmann et al 2005). In our own studies we have used the technique described here to detect levels of methylation for *ESR1* and *HPP1* of around 15%, and of around 0.3% for *p16* in samples of Barrett's oesophagus from human volunteers (unpublished observations). In the rat model, the mean percentage levels of methylation in both BO and OAC at 28 weeks after surgery were only 2.36% and 3.12% respectively in *ESR-1*, 1.68% and 0.11% respectively in *p16*, and 0.8% and 0.59 % respectively in *HPP1*.

There are a number of possible explanations for this difference. Technical difficulties dissecting both Barretts epithelium and tumour from the esophagus in the rat due to the tissue sample size, and intense degree of fibrosis related to the underlying inflammation, meant that the tissue samples may have contained elements of both the submocosa and muscular layers of the esophageal wall. When DNA was extracted from these samples any additional esophageal tissue in the sample would have also been processed, diluting the DNA from the Barrett's and tumour tissue. Indeed a dilution factor of between 2 and 5 would account for the difference observed between the methylation levels of *ESR-1* in the rat model and

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the reported findings of both Eads et al and our unpublished data in human Barrett's. Further, the levels of methylation in *ESR-1* were approximately 4 times higher in BO (2.4%) compared to oesophageal squamous epithelium (0.54%) in the rat. The difference in methylation levels between BO (14.9%) and oesophageal squamous epithelium (8.3%) we found in human volunteers (unpublished observations) was a similar magnitude. Similar differences in levels of methylation are also seen between the rat model and human disease in the *p16* promoter. However, this level of dilution would not account for the lower levels of *HPP1* methylation seen in the rat which are an order of magnitude lower than levels seen in humans. The proportional similarities in the levels of methylation in the two tissue types at the *ESR-1* and *p16* promoter between rat and humans would support the notion that the apparent differences in absolute methylation levels in rat and human are due to a dilutional effect. If this is the case, the lower levels of *HPP1* methylation suggest it may not be as important for disease progression in the rat.

Alternatively, the development of BO and OAC may occur independently of *ESR1*, *p16* and *HPP1* methylation in the model. There is a vigorous and extremely marked inflammatory response to the refluxate in rat oesophagus with rapid development of both BO and OAC. The intense inflammatory response may be sufficient to both initiate and drive carcinogenesis. The low levels of methylation that are observed in all 3 gene promoter regions 28 weeks after surgery may occur as a consequence of carcinogenesis in the rat oesophagus rather than contributing the process. In this case the low levels of methylation that occur in the rat would be

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unlikely to be of any biological importance in relation to gene expression. This would represent a potentially important inconsistency with the human disease.

5.1.3 GENE TRANSCRIPTION

Microarray studies allowed the expression levels of 14500 genes in the distal oesophagus of the rat model to be compared with gene expression levels in the distal oesophagus of non-operated age-matched controls. The number of genes undergoing transcriptional dysregulation in BO increased with time from surgery. The greatest number of differentially expressed genes was observed in OAC. There was reasonable homology in the pattern of altered gene expression amongst the 17 target genes identified as of key importance in the human disease process, with 13 (76%) of the 17 genes being over or under expressed in a similar fashion to the expression changes described in human OAC. In Barrett's tissue there was again good homology in the pattern of altered gene expression in the rat model compared to human disease. Nine (82%) of the 11 genes being over or under expressed occurred in a similar fashion to the expression changes described in human BO.

Like CpG island methylation, alterations in gene transcription and expression in human studies has demonstrated that only a proportion of patients with the disease will exhibit any given abnormality. Alterations in gene expression can occur as a result of genetic events such as aneuploidy, regional chromosome abnormalities, and point mutations within individual genes as well as a result of epigenetic events such as CpG island methylation. Certain genes commonly undergo transcriptional

deregulation, or have altered levels of expression, in Barrett's metaplasia and oesophageal adenocarcinoma. These include cell adhesion genes such as APC (Gonzalez et al 1997), the cadherin and catenin genes (Swami et al 1995, Bailey et al 1998), DCC (Wu et al 1998), and CD44 (Lagorce-Pages et al 1998); Cell cycle control genes such as CDK inhibitor p16 (Galipeau et al 1999), Rb (Barrett et al 1999), the cyclins D1 and E (Kataoka et al 1999, Lin et al 2000), and p21 (Hanas et al 1999); Tumour supressor genes p53 and p27 (Hamelin et al 1994); and genes involved in genome maintenance and the control of apoptosis such as mdm2 (Soslow et al 1999), Bcl2 (Rioux-Leclercq et al 1999), and the fas ligand. Indeed, no one single gene is vital for disease progression but rather it is the combined effect of all the genes that are affected in any individual that contributes to the development of carcinoma.

In our study we determined the global gene transcription profile of only three rats at each of the time points investigated. While reasonable homology in the gene expression profile in both metaplastic tissue and carcinoma was found between the rat model and human disease for those genes of interest represented on the microarray slides, a greater degree of homology may have been achieved if more replicates were studied. Owing to the small number of repilcates at each time point in the validation study, a meaningful analysis of variance with respect to gene transcription was not possible, and the similarities observed between the rat model and human disease are descriptive.

5.1.4 SUMMARY

In summary, the morphological changes, and the modifications to gene transcription that occur in the distal oesophagus of the rat following oesophagojejunostomy as it progresses from BO to OAC mirror those reported to

occur in human disease. However, they are not associated with high levels of methylation of *ESR1, p16,* and *HPP1* that are observed in the human disease. In general our findings support the use of oesophagojejunostomy in the rat as a model of human OAC in future chemoprevention studies. However, the low levels of CpG island methylation in the *ESR-1, p16,* and *HPP1* promoteers in this model raises the question as to the true mechanistic significance of CpG island methylation during the development of oesophageal adenocarcinoma both in the rat and in human disease. Further research on this issue, with a larger numbers of target genes, seems warranted.

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5.2 INTERVENTION STUDY

5.2.1 THE EFFECT OF ASPIRIN AND QUERCETIN

Having established and validated the surgical model of OAC in the rat as a model for human disease, the model was used to determine the efficacy of both the NSAID aspirin, and the bioflavonoid quercetin as potential chemopreventive agents in the prevention of OAC. The effects of the compounds on both disease initiation and progression were investigated in 2 separate studies. The first study was designed to determine the effect of these compounds on disease initiation. This was done by commencing treatment with either aspirin or quercetin 2 weeks before surgical induction of reflux disease. Treatment was then continued without interruption for the duration of the experiment. The results of this study were then compared with those of the second study in which treatment was commenced only after reflux disease had been firmly established in order to determine if treatment before the reflux induced injury influenced disease progression. From the results of the validation it was evident that all rats had severe oesophagitis and Barrett's oesophagus by 4 weeks after surgical induction of reflux. Treatment with aspirin or quercetin was therefore started at that time point in the second study and continued without interruption for the remainder of the experiment.

Treatment of rats with aspirin or quercetin before surgical induction of reflux disease had no effect on the proportion of rats that developed OAC compared to rats that received treatment once reflux disease was established. Three (25%) of the 12-surviving rats treated with aspirin prior to the onset of reflux disease developed

OAC compared to 10 of the 28 (36%) surviving rats treated after reflux was established (p=0.62). Similarly, within the quercetin group 6 (46%) of the 13-surviving rats treated with quercetin prior to the onset of reflux developed OAC compared to 14 (50%) of the 28-surviving rats treated after reflux was established (p=0.71). Further, all the rats that received either aspirin or quercetin prior to the onset of reflux developed Barrett's oesophagus.

The small numbers of~~in~~ rats involved in the pre-treatment groups of the intervention study make ~~a~~ it difficult to state with absolute certainty there was no reduction in cancer risk for those animals receiving treatment prior to the onset of reflux compared to those treated once reflux had been established. However, this would seem likely given that all animals fed both aspirin and quercetin prior to the onset of surgically induced reflux developed Barrett's oesophagus. This finding suggests that pre-~~-~~treatment does not influence the development of columnar metaplasia in the oesophagus in response to reflux induced injury in this model.

As the timing of treatment had no effect on the developed OAC in either the aspirin or quercetin group, the results for each compound from the pre-initiation and long-term study were combined in the overall analysis of their effect on the development of OAC. Overall, treatment with aspirin significantly reduced the incidence of OAC compared to controls. Thirteen (33%) of the 40 surviving rats that received aspirin developed OAC compared to 18 (60%) of the 30 surviving rats in the control group (p=0.003). A reduction in the incidence of OAC in quercetin treated

rats was also observed with 20 (49%) of 41 rats developing OAC compared to the control group. However, this reduction was not statistically significant (p=0.24). There was no significant difference in the incidence of BO in either the aspirin or quercetin treated groups compared to the control group.

This is the first in-vivo study to demonstrate that aspirin has a chemopreventive effect in the pathogenesis of OAC, and supports the epidemiological evidence which suggest that patients with any level of exposure to aspirin have a significant reduction in risk of developing both histological subtypes of oesophageal cancer. In the meta-analysis performed by Corley et al, frequent NSAID use was associated with a 43% reduction in risk of developing oesophageal cancer (Corley et al 2003). Subgroup analysis of both the histological type of cancer, and NSAID used, demonstrated that patients taking aspirin had a 33% reduction in risk of developing OAC. In the rat model, there was a significant reduction in the incidence of OAC in the aspirin treatment group compared to controls. This represented an absolute reduction in risk of developing OAC of 27.5% (4.7%, 50.3%), a similar risk reduction to that seen in human disease.

The lack of any demonstrable difference in the incidence of OAC between rats pre-treated with aspirin compared to those treated only once reflux disease was firmly established suggests that aspirin exerts its protective effect by inhibiting disease progression through the metaplasia – dysplasia - carcinoma sequence rather than by inhibiting disease initiation. This observation is supported by the finding that all

rats in the pre-treatment group developed Barrett's oesophagus indicating that aspirin has little or no inhibitory effect on the metaplastic transformation of oesophageal squamous epithelium in response to reflux induced injury.

The effect of quercetin on cancer pathogenesis in the rat model was less dramatic than aspirin. While there was a reduction in the incidence of OAC in rats treated with quercetin compared to controls, this did not reach statistical significance. Whether this trend represents a genuine protective effect that was missed because the number of rats in this arm of the study was too small is impossible is say. However, this study did find that all rats treated within quercetin in both the pre-treatment group and the group treated after the establishment of reflux disease developed Barrett's oesophagus. In addition, the proportion of rats that developed adenocarcinoma in both these groups was similar. If quercetin does have a protective effect in this model, it is also likely that it acts by inhibiting disease progression through the metaplasia – dysplasia - carcinoma sequence.

5.2.2 DRUG DOSING

The reduction in cancer incidence in rats treated with aspirin was achieved with a dose of 30mg/Kg/24h. This dosing regimen produced a mean plasma aspirin concentration of 18.9 mmol/L, confirming that aspirin was systemically absorbed. However, owing to the method of drug administration it is not possible to relate the plasma levels of aspirin in the rat to plasma levels that are achievable in humans with therapeutic doses of aspirin. Rats received a fixed dose of aspirin over a 24h

period. This was administered daily by mixing it in a 20g aliquot of food, the mean amount of food an adult rat will eat in a 24h period, which was fed to individually caged rats. Most rats ate most or all of the food each day. However, the food was ingested slowly throughout each 24h period resulting in a constant low dose administration of the drug. In addition, the blood samples taken for analysis of plasma aspirin concentrations were obtained when the rats were sacrificed. The timing of this event varied throughout the experiment resulting in blood samples being taken at different times of day. Consequently, even if the entire dose of aspirin was consumed at a given time, the random timing of blood sampling for each individual rat make the results difficult to compare.

Similarly a mean serum concentration of 0.24mg/L was achieved for quercetin and its metabolites with a dose of 70mg/Kg/24h confirming systemic absorption. Again, it is not possible to relate the plasma levels of quercetin to those achievable in humans because the dosing of quercetin, and the collection of blood samples for analysis, was performed in the same way as aspirin.

Given the uncertainty about the dosing levels achieved in the rat model with both aspirin and quercetin further studies in this area seem warranted. A controlled method of administering these drugs at a given dose and time of day, with appropriately timed sampling for measuring the blood levels achieved with any given dose, is necessary to assess if therapeutic levels have been achieved. An

even greater reduction in cancer incidence may be achievable with higher doses of aspirin and quercetin in this rat model with adequate therapeutic dosing.

5.2.3 CpG ISLAND HYPERMETHYLATION AND TREATMENT

Quantitative analysis of the levels of CpG island methylation in the promoter regions of *ESR-1*, *p16*, and *HPP1* in both treatment groups failed to demonstrate a significant difference in methylation levels in either Barrett's oesophagus or OAC compared to controls. However, there was a tendency to lower levels of hypermethylation in the *ESR-1* promoter in Barrett's oesophagus and OAC within both treatment groups. The reduction in methylation level was more pronounced in the quercetin group than the aspirin group in both tissues. Lower levels of hypermethylation were also seen in both treatment groups in the *p16* promoter region in Barrett's oesophagus, and in the *HPP1* promoter region in OAC. The reduction in hypermethylation was most pronounced in the quercetin group at the *p16* promoter, but aspirin seemed to have a greater effect at the *HPP1* allele at a level almost approaching statistical significance.

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The significance of reduced levels of CpG island hypermethylation in the treatment groups is uncertain. Lower levels of hypermethylation were associated with a lower incidence of OAC in both treatment groups, and the modulating effect of both aspirin and quercetin on the methylation levels at all 3 gene loci may account, at least in part, for this effect. However, a greater reduction in hypermethylation was seen in the quercetin group at 2 of the 3 gene loci examined, while the greatest

reduction in incidence of OAC was seen in the aspirin group. The disparity between the effect of the treatment on hypermethylation and disease development may indicate that methylation, at least at these 3 gene loci, is less important than other events in driving carcinogenesis in this model. Indeed, it is clear from the validation study that hypermethylation occurs at much lower levels in the rat model than seen in human disease at all 3 gene loci. If the low levels of methylation are a true reflection of the disease process in the rat, it is difficult to imagine how such low levels of hypermethylation could contribute significantly to cancer development and progression in this model.

Little is known about effect of NSAIDs or the flavonoids on either global hypomethylation or CpG island hypermethylation of the promoter regions of genes involved in cell growth during carcinogenesis in human OAC. However, the available published literature suggests both groups of compounds may exert an anti-cancer effect by modifying these epigenetic events.

Tao et al showed that global DNA hypomethylation in azoxymethane-induced colon tumours in male F344 rats was reversed by 7 days of treatment of sulindac, although low dose aspirin had no discernible effect (Tao et al 2004). The same group went on to demonstrate that the selective COX-2 inhibitor celecoxib could reverse global DNA hypomethylation in this model (Pereira et al 2004). Celecoxib was also shown to significantly reduce the number of hypermethylated CpG islands

within the promoter region of the *ESR-1* gene. However, no studies have been published that have quantitatively examined the effect of NSAIDs in OAC.

Similar modifications of both global hypomethylation and CpG island hypermethylation during carcinogenesis have been reported with some dietary polyphenols. Mittal et al reported that topical application of the flavanol epigallocatechin 3-gallate (EGCG) inhibited global DNA hypomethylation in mouse skin after exposure to UVB light (Mittal et al 2003). Further, both EGCG (Fang et al 2003) and the isoflavone genistein (Fang et al 2005) reversed CpG island hypermethylation in a number of genes implicated in carcinogenesis including *p16, hMLH1, RARβ* and *MGMT* in the human oesophageal squamous carcinoma cell line KYSE. Concurrent with the reduction in methylation level at the gene promoter, mRNA of all 4 genes, which had been undetectable, was detected following treatment. The effect of quercetin on methylation during carcinogenesis is less clear. Tao et al found treatment with quercetin produced no change in global DNA hypomethylation in azoxymethane-induced colon tumours in male F344 rats after 7 days (Tao et al 2004). However, a significant reduction of up to 70% in CpG island hypermethylation in the promoter region of *ESR-β, P16INK4a* and *RASSF1A* was shown in vitro by Ma et al in the human bladder cancer cell lines EJ, J82 and T24 after treatment with quercetin (Ma et al 2006). The effect of the polyphenols, and specifically quercetin, on the methylation changes that occur in OAC is unknown.

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The tendency to reduced levels of CpG island hypermethylation at all 3 gene loci in the rat model after treatment with both aspirin and quercetin is broadly consistent with the changes reported to occur during carcinogenesis in other in-vitro and in-vivo models. There are currently no published studies that have quantitatively assessed the effect of these or any other potential chemopreventive agents on CpG island methylation in human OAC. Given the current interest in methylation as a promotor of carcinogenesis, and the findings in this study of both reduced levels of OAC and CpG island methylation in the *ESR-1*, *p16*, and *HPP1* promoter regions, further studies in this area seem warranted.

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5.2.4 SUMMARY

In summary, treatment with aspirin was associated with a significant reduction in the incidence of OAC in the rat model, while treatment with quercetin was associated with a smaller non-significant reduction. Neither drug was effective in preventing BO suggesting that they exert a protective effect by inhibiting disease progression through the metaplasia – dysplasia - carcinoma sequence rather than by inhibiting disease initiation. This effect may in part be due to the ability of both aspirin and quercetin to reverse CpG island hypermethylation in the promoter regions of key genes implicated in carcinogenesis. This effect was apparent in the *ESR-~~esr~~ 1*, *p16* and *HPP1* promoters despite the relatively low levels of CpG island methylation that occur in the rat model. Further work on the ability of these agents to reverse CpG island methylation in OAC, a phenomenon not previously described, seems warranted.

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