ANIMAL MODELS OF LUNG CARCINOGENESIS AND CANCER CHEMOPREVENTION

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Lung cancer is the most important cause of cancer death. The epidemics of lung cancer is now declining in the male population of several countries but is still expanding in other countries, also including developing countries. These trends are surprising when considering that virtually all causes of lung cancer are known. Prevention strategies are based both on reduction of exposures to lung carcinogens, and especially to tobacco smoke, and on fortification of the host defenses by means of dietary and pharmacological agents. This approach, referred to as chemoprevention, exploits a variety of mechanisms that counteract tumour initiation, promotion, progression, invasion and metastasis (1).

The lung tumour assay is the most popular experimental test system to study chemopreventive agents. This model can be applied to evaluate inhibition of lung tumours, mainly adenomas, induced by individual carcinogens, among which several components of cigarette smoke (CS). For instance, we showed that administration with the diet of *N*-acetylcysteine (NAC), an analogue and precursor of reduced glutathione (GSH), prevents the formation of lung tumours induced by urethane in Swiss mice (2). Both NAC and the glucocorticoid budesonide inhibited the formation of lung tumours induced by benzo(*a*)pyrene in B16-129(F_1) mice, either wild-type or *fhit*+/– (3). Much more difficult is to reproduce in rodents the carcinogenicity of CS as a complex mixture. Witschi *et al.* (4) discovered that a weak but significant tumourigenic response can be elicited in A/J mice following whole-body exposure to environmental CS (ECS). We confirmed this finding in several experiments using A/J mice (5) and Swiss mice, even when exposed only throughout the pregnancy period and sacrificed 8.5 months later (6). This tumourigenic response was abolished in ECS-exposed mice receiving oral NAC during pregnancy (6). Although the lung tumourigenesis process is enhanced in (UL53-3 x A/J)F_1 mice carrying a dominant germline mutation of *p53* (7), in general the low yield of lung tumours in CS-exposed mice makes evaluation of chemopreventive agents difficult (4-6).

A great impulse to chemoprevention research has been offered by evaluating intermediate biomarkers in adult rodents as well as in critical periods of life, such as the prenatal period, birth and ageing. Thus, exposure to ECS of pregnant Swiss mice resulted in overexpression of 117 of the 746 tested genes in foetus liver. Co-treatment with NAC considerably reduced the number of overexpressed genes (8). In untreated mice, the sudden transition from the maternal-mediated respiration of foetuses to the autonomous respiration of newborn mice resulted in a 5-fold increase of DNA adducts, doubling of oxidative DNA damage, and upregulation of 33 genes in the lung. As an example of transplacental chemoprevention, treatment of dams with NAC prevented DNA and gene expression alterations in lung (9). A crucial event during ageing is represented by damage to mtDNA. Exposure of Sprague-Dawley rats to mainstream CS strongly induced formation of adducts to mtDNA, which were inhibited by the oral administration of NAC (10). Exposure of adult rats to ECS induced several alterations, including formation of DNA adducts in pulmonary alveolar macrophages (PAM), tracheal epithelium, lung, heart and aorta; oxidative DNA damage in lung; adducts to haemoglobin of 4-ABP and BPDE; cytogenetic damage in PAM and bone marrow; proliferation and apoptosis of PAM (11). These alterations were variously modulated by NAC, oltipraz (OPZ), 1,2-dithiol-3-thione (DTT), phenethyl isothiocyanate (PEITC), 5,6-benzoflavone (BF), and NAC plus OPZ

(11). Further studies investigated the regulation of 4,858 genes and of apoptosis in lung and liver of rats, either unexposed or exposed to ECS, treated with either NAC, OPZ, BF, PEITC, indole 3-carbinol (I3C), NAC plus OPZ, or PEITC + I3C (12). Chemopreventive agents should not excessively alter the baseline gene expression, as a molecular indicator of safety, while they should be able to attenuate CS-related alterations. This hypothesis was also validated at the proteome level by testing 518 proteins in lung (12).

The possible interactions between exposure to light and CS, which collectively are responsible for the 40% of human cancers, were investigated in SKH-1 hairless mice. The whole-body exposure of these mice to ECS produced a variety of molecular and biochemical alterations in the respiratory tract, skin, and bone marrow. Exposure to the light emitted by UV-C-filtered halogen lamps produced skin alterations, consistently with the potent skin carcinogenicity of these lamps (13). Surprisingly, the light alone induced formation of DNA adducts in lung and bone marrow, upregulated two genes in lung, and produced a systemic cytogenetic damage. Light and ECS had synergistic effects in the lung, which were attenuated by oral sulindac (14). On the whole, these and other data provide evidence that animal models are quite useful in exploring lung carcinogenesis, evaluating safety and efficacy of chemopreventive agents, and understanding the mechanisms involved.

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MODELS OF PROSTATE CANCER

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Purpose:
To review the availability of models for prostate cancer concentrating on nude-mouse models. Indications will be given about the potential usefulness of these models in exploring the basic aspects of prostate cancer as well as in their application to screening of new drugs and preventive agents.

Methods:
Models for prostate cancer have been derived by direct xeno-transplantation of human prostatic cancer tissue into nude mice. Mice with different genetic backgrounds have been used and have shown different take rates. Other models have become available through the vector immortalisation of cell lines. More recently, transgenic mouse models have been developed in several laboratories. In 1996 the first use of the nude mouse as a host for human tumour tissue was reported by Rygaard and Povlsen. Since then systematic attempts to utilise nude mice to establish immortal prostate cancer lines have been pursued. The PC82 line developed in the Rotterdam laboratory was the first one that found widespread application. This line is hormone dependent and produces PSA, which is measurable in serum. Since then a panel of 13 human prostatic xenograft lines have been developed in the Rotterdam laboratory and have become available for application. The characteristics of these lines will be described in detail.

While subcutaneous transplantation is the starting point, it was soon realised that other sites such as transplantation under the renal capsule and specifically orthotopic transplantation into the mouse prostate create more optimal growth and experimental conditions. A technique has been developed to monitor growth in the mouse prostate by transrectal ultrasonography. Correlation between tumour volumes and PSA determinations in blood taken from the orbital vain have been shown to be useful. With orthotopic transplantation sublines metastasising to lymph nodes and to the lung of one of the cell lines (PC346) could be developed. Also, the PC346 cell line was established in vitro and was shown to still be endocrine dependent, to produce PSA and to process the wild type androgen receptor.

Results:
The available lines, specifically PC82 have been widely used around the world to further explore aspects of endocrine dependence, progress to endocrine independence, identification and manipulation of growth controlling mechanisms of prostate cancer and other aspects. Furthermore, the lines have turned out to be useful in studying new drugs and preventive agents in a preclinical screening situation. Details on this experimentation will be referred to in another presentation.

MODELLING MAMMARY NEOPLASIA USING CONDITIONAL MOUSE MODELS.

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A series of transgenic mouse models of mammary neoplasia have been generated using either pronuclear injection or conventional gene targeting. One limitation to this approach has been the difficulty of analysing null mutations, as they often lead to embryonic lethality - as with both Brca1 and Brca2. This particular difficulty can now be overcome through the use of conditional alleles, for example using Cre-Lox technology. This approach has, for example, allowed models to be generated for both Brca1 and Brca2 deficiency. Using this approach, we have employed the ovine beta-lactoglobulin (BLG) promoter to drive Cre-mediated deletion of both Brca2 and Apc within the mammary epithelium (the latter resulting in activation of the Wnt pathway).

Deletion of Apc within the adult mammary epithelium results in a dramatic switch in the differentiation programme, resulting in squamous metaplasia. In the context of additional loss of either Tcf-1 or p53, we have also shown rapid progression of these metaplasias to neoplasia. The relevance of such models to human disease is only now becoming appreciated, as a hitherto unrecognised association between Wnt activation and mammary neoplasia has been revealed through epigenetic studies. Similarly, we have been able to delete Brca2 specifically from the mammary epithelium and show that this predisposes to mammary neoplasia- a process accelerated by p53 deficiency but not loss of either Apc or Stat3.

These models therefore allow an assessment of the genetic requirement for neoplasia. We are now also using them to test novel anti-tumour agents. One such family of agents are inhibitors of the DNA repair enzyme PARP. The logic used here is that tumour cells which arise in the context of failed DNA repair (eg following loss of Brca2) will be exquisitely sensitised to additional DNA damage, such as that arising following loss of PARP function. Inactivation of PARP is therefore hypothesised to lead to the specific deletion of tumour cells. We are currently testing this notion using mice deficient for Brca2 in both the mammary and intestinal epithelium. The use of the latter approach has allowed us to show that PARP inhibition rapidly and selectively kills Brca2 deficient cells, studies which we are now extending to the mammary gland to determine if PARP inhibition can act to either induce tumour regression directly or to function prophylactically.

In summary, these models are allowing us to identify new genetic mechanisms underlying mammary neoplasia, identify potential new chemotherapeutic targets and to rapidly test potential new therapeutics within an appropriate physiological setting.

COLON CARCINOGENESIS CHEMOPREVENTION: MIN MICE AND CARCINOGEN-INITIATED RATS, WHAT DO THEY TEACH US?

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Background: Epidemiology and in vitro results suggest potential chemopreventive agents, which are eventually tested in clinical trials (1). In between, they must be tested in preclinical studies (i) to know their in vivo efficacy, and (ii) to uncover mechanisms. Two major animal models of colon cancer are used: Min mice and carcinogen-initiated rats. This review compares these models with humans (2). Min mice have multiple intestinal neoplasia, due to a germline mutation of *Apc*, similar to that in many human cancers. The *Apc*(+/-) mice are thus promising models of human cancer (3), but the tumors occur mostly in the small intestine, not the colon (4). In rats, colon tumors observed after dimethylhydrazine or azoxymethane injections share many histopathologic and genetic characteristics with human tumors (5), but they accumulate beta-catenin due to *Ctnnb1* mutation, not due to *Apc* mutation (2).
Methods: To know if rodent models of colon carcinogenesis are good predictors of chemopreventive efficacy in humans, we made a meta-analysis of aspirin, beta-carotene, calcium, and wheat bran studies. Controlled intervention studies of adenoma recurrence in human volunteers were compared with chemoprevention studies of carcinogen-induced tumours in rats, and of polyps in Min (Apc(+/-)) mice: 6714 volunteers, 3911 rats and 458 mice were included in the meta-analyses. Full data, figures and global relative risks (RR) resulting from the meta-analysis approach are available online at http://corpet.net/min

Results: Differences between models was small since most global RR were between 0.76 and 1.00 (Fig.).

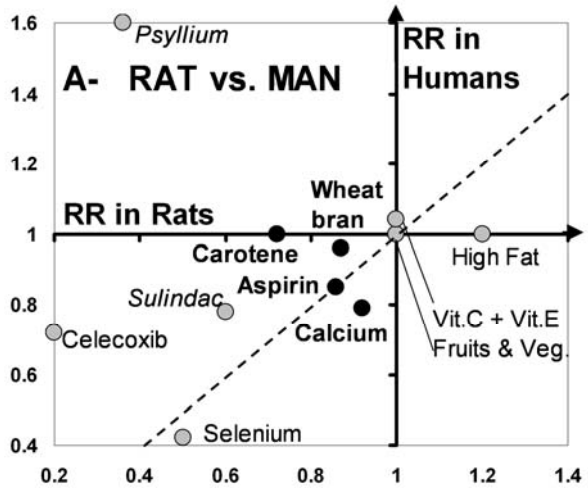
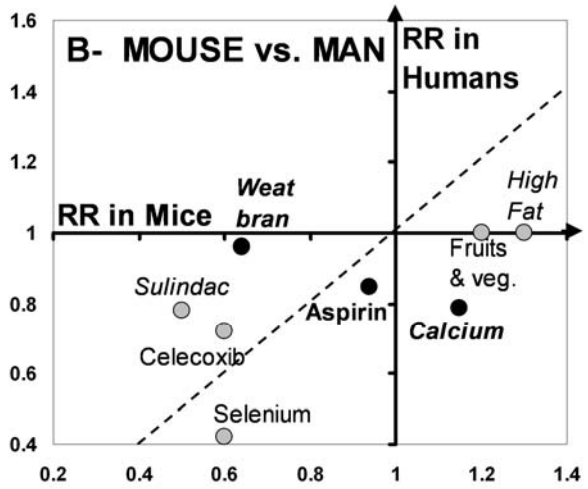


Figure: Chemoprevention Efficacy in Humans and Rodents, from the *Eur.J.Cancer* (2)
A- Colon polyp recurrence RR in humans vs. colon tumor RR in carcinogen-injected rats. **B**- Colon polyp recurrence RR in humans vs. Polyp Ratio in *Apc* mutated mice. Black points, meta-analysis data. Grey points, tentative values from review (4). *Italics*, RR significance discordant in humans and rodents.



A closer look shows that carcinogen-induced rat studies matched human trials for aspirin, calcium, carotene, and were compatible for wheat bran. Min mice results were compatible with human results for aspirin, but discordant for calcium and wheat bran (no carotene study in mice).

Conclusion: These results suggest that rodent models roughly predict effect in humans, but the prediction is not accurate for all agents, and the rat model might be better than the mouse model. Although none of the models reflects all aspects of human cancer, they complement each other to provide suitable tools for specific task (6). They are useful to screen potential chemopreventive agents, and to study mechanisms.

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DISCUSSION:
Which animal models are most useful in novel agent development?

XANTHOHUMOL FROM HOPS

NOVEL FLAVONOIDS

Andy Gescher

The consumption of naturally occurring flavonoids has long been suspected to exert beneficial health effects, among them the prevention of malignancies of the breast and colorectal tract (1). Prominent examples of potentially cancer chemopreventive flavonoid aglycones, which have undergone extensive experimental scrutiny, are quercetin, which is contained in onions, apples and wine, and genistein, found in soya. Both these agents have been documented to prevent carcinogen-induced carcinogenesis in rodents and can be considered "benchmark" flavonoids in the chemoprevention of experimental cancer. There are more than 4000 naturally occurring flavonoids, of which only a handful have thus far been examined extensively for cancer chemopreventive efficacy. Our group discovered the potential cancer chemopreventive activity of the rice bran constituent flavone tricin (4',5,7-trihydroxy-3',5'-dimethoxyflavone) (2). We showed recently that dietary tricin (custom-synthesized for us by the NCI Chemoprevention Branch under the auspices of the NCI RAPID Initiative) reduces adenoma load in the *Apc*^{Min} mouse. We also found tricin to be a potent inhibitor of the enzymes cyclooxygenase (COX)-1 and -2, thus mimicking to some extent aspirin (3). Intriguingly, rice bran, the dietary source of tricin, was also highly efficacious in reducing adenoma in *Apc*^{Min} mice.

Tricin has been compared with apigenin (4',5,7-trihydroxyflavone), a constituent of leafy vegetables, which is chemically closely related to tricin. Unlike tricin, apigenin was a poor inhibitor of COX activity. However, apigenin was able to down-regulate COX-2 expression in human-derived colon cancer cells, whilst tricin had only an ephemeral effect on COX-2 expression. These preliminary experiments are consistent with the notion that structural features of the flavonoid molecule strongly influence their pharmacodynamic behaviour. Specifically, the presence of methoxy substituents seems to impart COX-inhibitory properties. There is evidence in the literature, which tentatively supports this idea. In a comparison between quercetin and its methyl analogues tamarexetin (4'-O-methylquercetin) and isorhamnetin (3'-O-methylquercetin), the methylated cogeners were significantly better COX-2 inhibitors in cells than quercetin (4).

A property thought to militate against the medical use of flavonoids is their poor systemic bioavailability, caused mainly by their avid propensity to undergo conjugative metabolism. In preliminary experiments we found that tricin, on account of its two methoxy functions, undergoes much less rapid conjugation by human liver preparations than apigenin. Likewise, oral consumption of equal amounts of either tricin or apigenin furnished higher blood levels of the former compared to the latter. These initial insights suggest that structural features of the flavonoid molecule strongly influence their pharmacokinetic behaviour, in that the presence of methoxy moieties imparts superior bioavailability onto the flavone molecule.

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POLYPHENOLIC ANTIOXIDANTS

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The research programme of our group concentrates on the isolation, purification, identification and preliminary screening of the antioxidant capacity of polyphenolic compounds present in various matrices of the plant kingdom. Our research efforts are focused on plant products of the Mediterranean basin, African, Brazilian, and Chinese medicinal plants and also encompasses extensive studies on `waste´ products of Thai and Brazilian tropical fruits. Within the context of this presentation I shall however concentrate on phenolic compounds found almost exclusively in extravirgin olive oil and the olives from which they are derived. Phenolic compounds in extravirgin olive oils were first described by Montedoro et al., (1993). However definitive quantitation was not achieved until 2000 (Owen et al., (2000a-d); likewise for olives (Owen et al., 2003). In the framework of these experiments the identification and quantitation of lignans as a major proportion of the polyphenolic fraction was described for the first time. This is important because they are precursors of enterolignans produced in humans which are deemed to be positive modulators of breast cancer risk (Knust, 2005). All polyphenolic compounds within extracts of olive and olive oil are more potent antioxidants than e.g. the tocopherols. However, of extreme interest is that oleocanthal (the di-aldehydic form of deacetoxy-ligstroside) first quantitated in olive oil by Owen et al., (2000a) was shown very recently to be a 3-4 times more potent inhibitor of Cox-1 and Cox-2 than ibuprofen (Beauchamp et al., 2005). These enzymes are regarded to be important in the aetiology of many cancers because they catalyse the steps in the biochemical inflammation pathways derived from arachidonic acid. The data indicates that more comprehensive screening of the potential cancer chemopreventive effects of other polyphenolic compounds in olives and olive oil is warranted.

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LYCOPENE: ANTIOXIDANT AND NON-ANTIOXIDANT PROPERTIES

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Lycopene is a major carotenoid in human blood and tissues but not a precursor of vitamin A like ß-carotene. The most important dietary sources for lycopene are tomatoes and all kinds of tomato products. As for all carotenoids, its bioavailability depends on food preparation, processing, and additional intake of dietary fat. Epidemiological studies suggest a correlation between an increased intake of lycopene rich food and a decreased risk for some cancers and cardiovascular diseases. At present, most promising epidemiological data point at a preventive effect of a lycopene-rich diet in context with protection against prostate cancer. The idea is supported by the biokinetics of lycopene and several in-vitro studies on the growth of cancer cells. However, there are still many questions open regarding protective effects of lycopene, respectively lycopene-rich products. It has yet not proven that lycopene is really the active compound; the mechanism(s) of action are still unknown. Lycopene is a very efficient antioxidant scavenging singlet molecular oxygen and peroxyl radicals. It acts synergistically with other compounds of the antioxidant network. The antioxidant properties have been discussed as a principle of protection preventing oxidative damage to the DNA. However, lycopene and several other carotenoids exhibit biological activies which are not related to their antioxidant potential and might play a role in cancer prevention. Carotenoids affect intercellular communication pathways, cell cycle progression, and gene expression. Bioactive metabolites or decomposition products are supposed to act as ligands for ligand-dependent transcription factors regulating gene expression.

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GLUCOSINOLATE BREAKDOWN PRODUCTS IN COLORECTAL CANCER PREVENTION

Ian Johnson

Glucosinolates, of which more than 100 are known to exist, are sulphur-containing glucosides found plants of the order Capparales (1). This group includes the brassica vegetables, and widely used condiments such as mustard and horseradish. Epidemiological evidence indicates that a high consumption of plant foods rich in glucosinolate breakdown products protects against carcinomas, especially of the gastrointestinal tract and lung. Glucosinolates are stable in intact plant tissue, but physical damage brings them into contact with the endogenous enzyme myrosinase (thioglucoside glycohydrolase EC 3.2.3.1), causing the release of glucose and a variety of active products, including the isothiocyanates. Food processing and preparation causes glucosinolates to be degraded or leached from vegetables, but consumption of raw vegetables, or thermal inactivation of myrosinase during cooking, leads to consumption of intact glucosinolates. Once ingested, intact glucosinolates are degraded, either by myrosinase from raw vegetables during digestion, or by bacterial myrosinase in the colon. The bioavailability of glucosinolate breakdown products is highest from raw vegetables. For example, metabolites derived from isothiocyanates have been shown to be detectable in the urine 2-3 hours after consumption of a meal containing raw broccoli.

Isothiocyanates are potent inducers of Phase II enzymes in vitro. They have also been shown to increase the metabolism and detoxification of chemical carcinogens in both animal models, and in human subjects. Recent evidence shows that the consumption of brassica vegetables is associated with a reduced risk of lung cancer (2) and colorectal polyps (3) in subjects with common polymorphisms of glutathione-S-transferase (GSTT -/- & GSTM1-/-). This suggests that a slow rate of isothiocyanate metabolism may confer a biological advantage, but raises questions as to the mechanism of action. A plausible alternative mechanism for the protective effect of glucosinolate breakdown products is the suppression of neoplasia by induction of apoptosis in epithelial tissues. Isothiocyanates and other glucosinolate breakdown products from brassica tissues inhibit cell proliferation by blocking the cell cycle in human tumour cells in vitro (4) . Consumption of brassica vegetables leads to inhibition of mitosis and an enhanced rate of apoptosis in crypt epithelial cells of the rat colon, following challenge with a chemical carcinogen (5). Glucosinolate breakdown products are potentially toxic and may best be provided for human consumption in the form of high glucosinolate vegetables. A glucosinolate-rich variety of broccoli has already been developed and is undergoing clinical trials (6).

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COLON CANCER CHEMOPREVENTION BY DIETARY PEG

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Introduction:
Polyethylene-glycol 8000 (PEG) is a water-soluble polymer: CH3-(O-CH2-CH2)n-OH. PEGs of high MW are not absorbed, but attract water in the gut. They cause an osmotic mild laxative effect, similar to fibers effect. We made the hypothesis that PEG might reduce colon carcinogenesis, and give here a review of experimental studies on PEG chemoprevention.
Materials and Methods: The hypothesis that PEG might protect rodents against chemically-induced carcinogenesis was tested in 25 independent studies, involving more than 1000 animals. The general design was to initiate carcinogenesis by azoxymethane injection, then to randomize rats (or mice) to diets containing 5% PEG or no PEG. Most studies lasted for 30 days with the aberrant crypt foci (ACF) endpoint. Adenomas and carcinomas were looked for in 3 long-term studies. Two studies of PEG effect in mutant mice were performed in American institutes. Last, a human population-based study tested if the use of PEG-based laxatives is associated with reduced tumor risk.

Results:
ACF studies showed that PEG had a strikingly potent effect. PEG-fed rats have 100 times fewer large ACF than controls ($p<0.0001$). Some treated rats have no detectable ACF, a protection never observed before (1). PEG protection is time- and dose-dependant (2), and a 3-day PEG treatment halves the ACF number (3). PEG is active in rats and mice, and suppresses ACF initiated by various carcinogens including AOM, DMH, MNU and MeIQ. All high MW PEGs suppress ACF, the optimum being observed for PEG 8000 (3).

Cancer studies: Dietary PEG decreases the colon cancer incidence in AOM-initiated rats from 19/27 to 2/21 and the multiplicity from 3.1 to 0.3 tumor/rat (both $p<0.0001$) (2). In a second study, PEG decreased 7-fold the tumor incidence and 20-fold the tumor multiplicity (both $p<0.005$) (3). A third cancer study showed that a 30-day PEG treatment, that was started 5 months after carcinogen, shrank established polyps and halved total tumor load ($p=0.02$) (unpublished observations).

Min mice studies: Two studies of PEG effect on tumor in Min mice yielded opposite results. In a first lab, PEG treatment significantly promoted tumors in the colon of mice (4). In contrast, in a second lab, PEG treatment afforded a significant protection in the small intestine of Min mice (5).

Epidemiology: Among 1165 patients undergoing colonoscopy near Tours (France, Indre-et-Loire), previous Forlax intake was more common in tumor-free patients (OR=0.52, $p=0.03$), which suggests that Forlax®, a PEG-based laxative, may halve the risk of adenoma (6).

Mechanistic studies: When PEG is added on top of cancer cells in vitro, their growth is fully inhibited. Non-cancer cells are not inhibited (7). PEG also induces apoptosis and pro-apoptotic factor Par-4, and suppresses E-cadherin down-regulating factor SNAIL (8-9). Last, PEG reduces inflammation in the colon of carcinogen-initiated rats (10).

Conclusion:
PEG is a fast and very potent inhibitor of carcinogenesis in rats. No other agent is more potent than PEG to prevent ACF except PEG-like pluronic F68 (11). PEG is also strikingly potent to inhibit cancer formation: it is second only to celecoxib in the standard AOM/rat model (cf. Chemoprevention database http://corpet.net/min). PEG is not absorbed from the intestine, it is tasteless and a high-grade purity can be produced at low cost. PEG has a long history of safe use in humans, and has no known toxicity: we think PEG effect should be tested in a human volunteers clinical trial.

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ANTI-ANGIOGENESIS IN A CHEMOPREVENTIVE SETTING: THE RATIONAL OF ''ANGIOPREVENTION''

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Angiogenesis inhibition as an approach to the treatment of solid tumors based on the rationale that, when deprived of vascularization, most tumors cannot expand. If we could prevent the “angiogenic switch”, we can prevent progression of hyperplastic foci, cancer insurgence and metastasis. The principle of cancer chemoprevention is based on the use of agents that, while devoid of collateral effects, are able to interfere with processes associated with malignant progression. In working with many chemoprevention agents, we recently observed that angiogenesis is a common a key target of most chemopreventive molecules. We termed "Angioprevention" the concept that effective chemoprevention targets angiogenesis (1). We have mostly concentrated on the radical scavenger N-acetyl-L-cysteine (NAC) and the green tea flavonoid epigallocatechin gallate (EGCG). Both of these molecules inhibit the matrix metalloproteinases MMP-2 and MMP-9, blocking tumor cell invasion in vitro, and appear to interfere with VEGF production or VEGF signaling as well. In addition, they significantly inhibited growth of the vascular tumor Kaposi’s sarcoma xenografts in vivo.

The concept of "angioprevention" can be extended to other chemopreventive molecules, including natural or synthetic retinoids such as 4-hydroxyphenylretinamide - fenretinide (4-HPR). 4HPR inhibits endothelial cell growth, morphogenesis, chemotaxis and invasion, and in vivo 4HPR inhibited angiogenesis in the Matrigel plug assay. Inhibition of invasion by 4HPR was associated with a decreased release of MMP-2.

In order to find other molecular mediators of the anti-angiogenic effects we have performed microarray expression profiling of endothelial cells in response to angiopreventive molecules using the Affymetrix GeneChipTM platform. NAC and EGCG show similar but distinguishable effects on human umbilical endothelial cells (HUVEC). Consistent with the absence of toxicity, no drastic changes in gene expression were observed. Many of the genes that respond to both drugs are annotated as involved in angiogenesis related processes and give direct clues to the observed angiopreventive effect since endothelial cell migration, cohesion and adhesion, viability and apoptosis are affected. The overall picture that emerges from the detailed functional annotation is that the anti-oxidants stabilize the endothelial cell in a less proliferative and less apoptosis-prone state with a reduced migratory potential (2). The synthetic retinoid 4HPR followed a different pathway of regulation linked to expression of anti-angiogenic molecules in TGF-beta pathway, MIC1 and BMP2 (3).
Besides tumor-entothelial cell interactions, innate inflammatory cells appear to often play a key role in assisting tumor growth, expansion and angiogenesis. Thus compounds that can repress tumor-endothelial cell and/or inflammation will show promise in the chemoprevention setting.

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THE CHEMOPREVENTIVE AGENT DEVELOPMENT RESEARCH PROGRAM IN THE U.S. NATIONAL CANCER INSTITUTE – RECENT ACHIEVEMENTS AND CURRENT ACTIVITIES.

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Over the past 25 years the Division of Cancer Prevention (DCP) of the U.S. National Cancer Institute has organized a research and development program to provide resources and infrastructure to the research community for the clinical evaluation of potential cancer preventive agents. Several successful phase 3 cancer prevention trials have been supported by the DCP during this time. The earlier stages of the program now encompass preclinical agent and molecular target identification, in vitro and in vivo screening, efficacy and intermediate endpoint testing, pharmacology and toxicology assessments, and finally chemical synthesis and manufacturing leading to Investigational New Drug applications and clinical studies. In this overview, examples of agents currently in development, particularly those modulating the AKT pathway, will be described. The most commonly used preclinical in vivo animal models for testing efficacy, as well as newer models in development, will be presented, as will current phase 1 and 2 clinical studies.

The RAPID program within the DCP provides opportunities for international collaboration in the early stages of agent development. The program assists academically affiliated investigators bring novel compounds and concepts to clinical testing by providing product scale-up, conducting toxicology studies required by regulatory authorities, and filling other needs of applied drug development. Several international projects are being supported such as the manufacture of an HPV-16 capsid protein vaccine in India. Other collaborations with international pharmaceutical and nutritional manufacturers will also be cited.

New areas of technology development are also being explored for their application to preventive agent development. These include computer assisted drug design and cell systems biology. Finally, the NCI and the U.S. Food and Drug Administration are collaboratively developing new guidances and procedures to support chemopreventive agent testing. The continued commitment to cancer prevention research will significantly reduce the economic and medical burden of cancer.

DISCUSSION:

The European dimension: How can we better co-ordinate and optimise cancer chemopreventive agent discovery and development in Europe? Can the NCI help? How could we collaborate?

CURRENT STATUS OF TRIALS LOOKING AT SERMS AND AROMATASE INHIBITORS

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Selective estrogen receptor modulators (SERMs) play a key role in breast cancer chemoprevention. These agents antagonize estrogens in some tissues and mimic their action in others. The mechanism for tissue selectivity appears to be related to differences in their molecular and three-dimensional structures, which affect the transcriptional activity of the activated estrogen receptor.

For example, tamoxifen and toremifene act as estrogen antagonists in breast tissue and as estrogen agonists in the endometrium. Conversely, raloxifene behaves as an estrogen antagonist in both the breast and the endometrium. Studies of tamoxifen have proved that chemoprevention can successfully cover all three settings of prevention: a) primary chemoprevention, as shown in the NSABP P-1 trial in healthy women at increased risk according to the Gail's model (1); b) secondary chemoprevention, as described in the NSABP B-24 trial, in which patients with ductal carcinoma in situ (DCIS) benefited from tamoxifen for prevention of ipsilateral and contralateral breast cancer (2); and c) tertiary chemoprevention, as demonstrated in the EBCTCG metanalysis, wherein tamoxifen was associated with prevention of contralateral breast cancer in definitively treated breast cancer patients (3). Based on the results of the NSABP P-1 study, the Food and Drugs Administration has approved the use of tamoxifen as a chemopreventive agent in women with individual risk >1.66% in 5 years according to the Gail's model, which includes age, age at menarche, age at first pregnancy, first degree family history of breast cancer and number of biopsies for benign disease, with or without atypical hyperplasia (http://bcra.nci.nih.gov/brc/).

However, tamoxifen causes undesirable side effects, including an increased risk of endometrial cancer and venous thromboembolic events (VTE). Other SERMs are therefore entering the field of clinical cancer prevention and a large number of agents is likely to be tested in the next few years. The second generation SERM raloxifene is currently undergoing evaluation in comparison with tamoxifen in a large phase-III trial. Also, lower doses of tamoxifen are being assessed in phase II-III trials. In addition, the combination of hormone replacement therapy (HRT) or aromatase inhibitors and tamoxifen at low doses may reduce the risks while retaining the benefits of either agents. Finally, new agents that interfere with the onset of ER-negative breast cancer are being sought for combination chemoprevention since almost a third of breast cancers will not be sensitive to hormonal modulation.

The role of the third generation aromatase inhibitors is also being actively investigated in breast cancer chemoprevention due to important findings from secondary analyses in three major trials conducted in the adjuvant setting. The ATAC trial (4), the IES trial (5) and the MA-17 trial (6) evaluated the role of anastrozole, exemestane and letrozole, respectively, in the adjuvant therapy for early breast cancer in postmenopausal women. Primary endpoint was disease-free survival (DFS) and all three agents conferred a DFS advantage compared to standard, tamoxifen-based, adjuvant treatment. Analyses of secondary endpoints revealed that the aromatase inhibitors significantly decreased the incidence of contralateral breast cancer

compared to standard adjuvant therapy (42% risk reduction for anastrozole, 95% CI, 0.12-0.62, p=0.01; 56% risk reduction for exemestane, 9 versus 20 cases, p = 0.04; 46% risk reduction for letrozole, 14 versus 26 cases, p<0.01) (4,6). Given the encouraging results on contralateral breast cancer risk, aromatase inhibitors hold great promise as chemopreventive agents and the results of several ongoing trials are awaited with interest. For instance, the IBIS-II trial is comparing anastrozole to placebo in postmenopausal women at increased risk of breast cancer. However, the long-term safety of these agents remains an issue as these agents increase the risk of bone fracture and possibly cardiovascular disease.

Despite the encouraging results of the clinical trials, several important questions await resolution, including the appropriate duration and dose of tamoxifen, concomitant use of aspirin, development of new SERMs, assessment of long-term efficacy and safety of aromatase inhibitors, a better definition of subjects at increased risk for ER-positive breast cancer, the exclusion of subjects at risk for adverse events and the discovery of agents with preventive activity in ER-negative cancers.

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CHEMOPREVENTION OF LUNG CANCER

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Among all cancer, lung cancer has the highest rate of mortality in the western world. The poor lung cancer survival figures argue powerfully for new approaches to control this disease such as chemoprevention, that has been defined as the use of agents that reverse, suppress or prevent lung carcinogenesis.

Smoking

Over 80% of lung cancers are attributed to tobacco and carcinogens from cigarette smoke form the unquestionable link between nicotine addiction and lung cancer. Confoundingly epidemiological studies show that not more than 15% of heavy smokers will ultimately develop lung cancer. Dietary and genetically determined factors play an important role in modulating individual susceptibility and are closely linked to the chemoprevention approach. In case-control studies defective repair of genetic damage and increased sensitivity to mutagens have been associated with increased susceptibility to lung cancer. Also the DNA excision repair pathway to influences the individual susceptibility for lung cancer. From molecular studies it is known that genetic alterations persist after the cessation of tobacco use. In this respect it should be reminded that ex-smokers carry an elevated risk of lung cancer for many years and that most of those who now develop lung cancer in the US are ex-smokers.

Diet

The relationship between diet and lung cancer has been extensively explored in many ecologic and case-control studies and there are many leads to support an association between a high intake of fruits and vegetables and a reduced risk of lung cancer. In the past attention has focused on the pro-vitamin A carotenoids, particularly beta-carotene because of their antioxidant properties and the importance of vitamin A in cell growth and differentiation. More recently other micronutrients have been identified as having the potential to decrease lung cancer risk, including vitamin E, selenium, isothiocyanates, allyl sulphur compounds and green tea polyphenols. Isothiocyanates are non-nutrient compounds in cruciferous vegetables that can influence P-450 enzyme levels and enhance detoxification. A recent series of newly diagnosed lung cancer cases had significantly lower isothiocyanate intake when compared with controls. In this study also glutathione-S-transferase (GST) (null) genotype and smoking were associated with increased lung cancer risk, suggesting that smokers with low intake of isothiocyanates and a null GST genotype carry an extra risk. Allyl sulphur compounds that may induce apoptosis, and green tea anti-oxidants (polyphenols) have also been mentioned as potential preventive agents.
Overall, the epidemiological data support the hypothesis that a different intake of dietary compounds could modulate the risk of lung cancer. The confirmation of this hypothesis, however, can only be provided by carefully designed prospective trials that balance for smoking behaviour and ideally also take diet-gene interactions into account.

Multistep Carcinogenesis

The rationale for chemoprevention is based on two principles of tumor biology common to most epithelial cancers: field cancerization and multistep carcinogenesis. Field cancerization was first described by Slaughter et al in 1953, who identified epithelial hyperplasia hyperkeratinization, atypia and also carcinoma in situ in grossly

normal appearing epithelium adjacent to cancers of the oral cavity. These histologic changes throughout the oral epithelium suggested that the development of malignancy in the aerodigestive tract is not a random event but rather the result of diffuse changes that are present throughout the epithelium. The existence of diffuse premalignant changes in the aerodigestive tract confirms the observation that patients who survive a first cancer in this region are subsequently susceptible to the development of a second primary tumor .

Specific genetic alterations seem to be involved. For example, it has been shown that highly specific deletions in the short arm of chromosome 3 occur during hyperplasia, a very early stage on the way to lung cancer. Although the series of events eventually leading to the development of lung cancer are not yet fully elucidated, it is generally accepted that lung cancer develops in a stepwise fashion as the result of multiple genetic events. Suppressing one or more of the pre-invasive steps may impede the development of cancer.

Retinoids

Several randomized clinical chemoprevention trials have been completed in the 1990s. Among these the Alpha Tocopherol Beta Carotene (ATBC) and the Beta Carotene and Retinol Efficacy (CARET) trials. The ATBC trial accrued men, all heavy smokers and tested the effects of dietary supplementation of beta-carotene and alpha-tocopherol. Against all expectations this trial did not show any protective effect from either alpha-tocopherol or beta-carotene. On the contrary, beta-carotene was associated with a statistically significant increase (18%) in lung cancer incidence and mortality (8%). The detrimental effect of beta-carotene was confirmed by the CARET study, also involving smokers. CARET revealed a 28% higher rate of lung cancer and 17% higher overall death rate in those participants taking beta carotene. The findings of ATBC and CARET have clearly been a shock to the scientificn community but they have shown the unquestionable importance of large-scale controlled (randomized) studies. Since the detrimental effect of beta-carotene was seen primarily in smokers, it has been hypothesised that cigarette smoke in the lungs , which is highly oxidizing, may interact with beta-carotene, yielding unstable by-products that could have pro-oxidant activity. An experimental study in ferrets exposed to cigarette smoke and beta-carotene has suggested that such an interaction exists.

Second Primary Tumours

One of the earliest studies with relevance to lung cancer was a trial in 103 patients with previous head & neck cancer in which the effect of 13-cis retinoic acid on recurrence and second primary cancer was studied. The incidence of second primary tumors was significantly lower in the treatment arm (4% versus 24%). The second trial involved 307 patients with early stage lung cancer, randomized after complete surgical resection to receive either retinyl palmitate or no further treatment. Also in this study there was a significant difference in the frequency of second primaries (12% versus 21%).

The Euroscan study designed to assess the effects of retinyl palmitate and the antioxidant N-acetylcysteine in almost 2600 patients with early stage head and neck cancer or lung cancer following treatment with curative intent, did not confirm the positive outcomes of these previous studies. A similar result was obtained in the US NCI intergroup trial with 13 cis-retinoic acid to prevent second primary tumors (SPT) in Stage I NSCLC. 13 cis -retinoic acid did not impove the rate of SPT's or mortality .Subgroup analyses suggested that 13 cis-retinoic acid might have been harmful in smoking patients and beneficial for those patients belonging to the category of never-smokers. The Euroscan and the NCI intergroup trials underline the importance of large confirmatory trials. Both studies also strengthened the importance of smoking

cessation: participants who had permanently stopped smoking had a better survival than those who continued smoking.

New classes of agents

Advances in molecular biology has led to a better understanding and knowledge about important pathways necessary for cancer development, and antibodies and small molecules have been developed to target specific proteins and block important signaling pathways. Some of these molecules have attracted attention as potential chemopreventive agents. The identification of the ErbB-family (Epidermal Growth Factor Receptor (EGFR), Her-2/neu, HER-3 and HER-4) as a family of receptors has been especially important. These receptors activate after ligand binding a cascade of biological- and physiologically reactions eventually leading to cell proliferation and apoptosis. These signaling pathways can be blocked at different levels. Likewise, activation of the EGFR involves RAS activation. Farnesyl transferase inhibitors (FTI) block the RAS pathway and are also considered as potentially chemopreventive. Similarly the prostacyclin and the eicosanoid pathways, highly involved in carcinogenesis have been identified as targets for chemoprevention. COX-inhibitors, preventive in the digestive tract, are now studied in person's at risk for lung cancer. Today's challenge is to find the most optimal therapy (or combination of therapies) given the fact that certain requirements for feasibility and low level of toxicity are needed for "healthy" populations at risk for lung cancer. Reflecting on the multitude of potential chemopreventive agents ready for phase I and II testing it cannot be underlined enough that robust models for early clinical response evaluation are essential.

Conclusion

The understanding of the molecular and the biological mechanisms of lung cancer development has significantly expanded over the last 10 years. Multiple genetic and biological events have been identified that are considered essential for the carcinogenic process leading to the final malignant phenotype. Some of these lesions have been found closely associated with tobacco smoke exposure, and its has become increasingly clear that there is a wide variation in individual susceptibility to lung cancer. Valid risk markers are now appearing on the horizon, and a large number of novel agents with chemopreventive potential has been identified including molecules that block cellular receptors and important signaling pathways,

It is anticipated that after a long period with increasing insight into lung carcinogenesis but without appreciable benefits of the chemoprevention approach in randomized trials, the road is now being paved for more successful studies in high-risk individuals. Considering the continuing lung cancer epidemic worldwide it is clear that we must continue our effort to strengthen the fundamental strategy of avoidance the exposure of carcinogens in parallel with the development of additional approaches such as chemoprevention.

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PAST AND CURRENT CLINICAL STUDIES OF PROSTATE CANCER IN NUDE MOUSE MODELS (NEW DRUGS, PREVENTIVE AGENTS)

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Purpose

To show the usefulness of nude mouse models in new drug development and in the development of potentially active preventive agents for prostate cancer.

Methodology

- Orthotopic transplantation of the PC346 cell line into nude mice.
- Monitoring of tumour growth in the prostate and/of PSA in blood taken from the orbital vain of the nude mouse.
- Application to a self-composed cocktail with potential as a dietary supplement, lycopene plus Vitamin E and a soy extract.
- Data on a study of Iressa® will also be presented.
- The methodological concept: A clinical phase II study based on PSA kinetics is combined with a study in nude mouse which contrary to the clinical study allows measurement of tumour volumes and of PSA.
- An algorithm for evaluation has been developed and will be demonstrated.

Results

One of the clinical studies, the study of Iressa® has shown negative results in the clinical and in the experimental setting. Possibilities of how these results will influence the decision and development process for further evaluation of Iressa® in prostate cancer will be discussed. The 3 preventive studies will be reported in detail. As yet, for none of these studies both, the clinical and the experimental arm have been completed. Clinical effects of the self-made dietary supplement and of the study related to a potentially marketable soy extracts have been observed with respect to a prolongation of the doubling time of PSA. The Vitamin E / lycopene study has been completed in animals and has shown effects on tumour growth and Vitamin E.

Conclusions

Nude mouse models are useful in studying the effects of new agents or preventive agents in combination with related clinical protocols. Such studies allow dose finding, the study of combinations of agents and other important aspects of new treatments and of prevention short of initiating very expensive and rigid phase III protocols.

COLORECTAL CANCER: WHAT CAN WE LEARN FROM PAST AND CURRENT CLINICAL STUDIES OF NSAIDS AND COX-2 INHIBITORS?

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Patients with previous colorectal cancer (CRC) or precursors (adenomas) usually are followed with colonoscopy and polypectomy to reduce incidence of CRC as well as mortality from CRC. Further intervention by chemoprevention has been suggested but is not widely used. The effects of NSAID are many, but inhibition of synthesis of prostaglandins has been given most attention. Many case-control and cohort studies have suggested decreased formation of adenomas as well as CRC and RCTs have confirmed the inhibition of adenoma formation, but not the decrease in CRC incidence, which may partly be explained by the short term follow-up. Dose-relationship is not firmly established. The number of adenomas in patients with FAP is reduced and case-control and cohort studies in patients with IBD suggest a decreased risk of CRC.

The most important sideeffect of NSAID is peptic ulcer bleeding, but haemorrhagic stroke has also been found more often than among controls in RCTs. Colonoscopy surveillance every 10 years and every 3 years in patients with previous adenomas seem more cost effective than 325 mg Aspirin daily. The same is probably true for screening the average risk population with faecal occult blood tests followed by colonoscopy in those with positive tests.

Selective COX-2 inhibitors have been shown to have a protective effect towards adenoma formation in observational studies and RCTs without any increased risk of peptic ulcer bleeding. However, severe cardiovascular side affects have been registered and selective COX-2 inhibitors should only be considered in patients with high risk of getting CRC, high risk of peptic ulcer bleeding and no increased risk of cardiovascular disease.

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EARLY CLINICAL TRIALS WITH POLYPHENOLS

William P Steward

There is increasing evidence that diet-derived constituents may play a role in cancer prevention. Diet constituents which have been shown to have promise as chemopreventives from epidemiological studies are purified and examined in preclinical models for potential mechanisms of efficacy. Transgenic models of carcinogenesis may also support the choice of such agents for future human clinical trials.

Phytochemicals form the focus of preclinical and early clinical development within the Chemoprevention and Biomarkers Group in Leicester and agents which are currently being examined include black and green tea extracts, the curry constituent curcumin, tricin (derived from rice), silibin from milk thistle and resveratrol (from grapes and red berries). These agents have been demonstrated to have mechanisms of action which support their development as chemopreventives and also have activity at delaying the onset of tumours in transgenic models.

Curcumin, a major constituent of the spice turmeric, inhibits the activities of protein kinase C, epidermal growth factor receptor kinase, oncoprotein tyrosine kinase p185, nitrogen-activated protein kinases, and c-Jun terminal kinase. Curcumin has also been found to affect reactive oxygen species and to inhibit expression of the gene for nitric oxide synthase. A further important potential mechanism for this agent is the inhibition of COX-2. From an analysis of preclinical mechanisms, it appeared that curcumin may have potential in chemoprevention and its effect was examined in the Min mouse model in which it significantly reduced small bowel adenoma formation. Given this activity, curcumin was taken forward into phase I studies in Leicester. The first of these involved patients with colorectal malignancy who took daily doses of up to 100 mg per day for four weeks. This was well tolerated (1). No systemic bioavailability was demonstrated and doses were subsequently increased in a further cohort of 15 patients up to a maximum of 3.6 grams per day for up to four months. At the top dose level, traces of curcumin and its conjugates were detected in plasma and urine and there was a significant reduction in inducible PGE_2 in the blood. No toxicity was observed (2). In order to obtain more detailed pharmacokinetic and pharmacodynamic information, a pre-surgery model was utilised with patients who have been diagnosed with patients who had been diagnosed with colorectal cancer and who were awaiting colectomy. Diagnostic biopsies were obtained and patients received one week of daily curcumin at doses between 0.45 and 3.6 gms per day. The last dose was taken prior to surgical resection and tissue, blood and bowel were obtained. Tissue levels of curcumin within the resected colon were $0.8\text{-}1.3 \times 10^{-8}$mol/g. There is an indication of an antioxidant effect from measures of M_1G levels before and after exposure to curcumin. Again no toxicity was observed (3). A further 12 patients who were undergoing hepatic resection for liver metastases from colorectal cancer also took curcumin for seven days prior to liver resection. No curcumin was detected within the liver tissue and only trace levels were measured as conjugates in the portal circulation (4). From these phase I studies, it appears that curcumin is safe and well tolerated for up to 4 months continuous administration at doses up to 3.6g per day. These dose levels resulted in extremely low systemic bioavailability but sufficient concentrations within colorectal tissue to exert a biological effect by extrapolation from preclinical models. The demonstration of an antioxidant effect within colon tissue indicates the potential for curcumin as a chemopreventive. Further studies are now planned in patients with familial adenomatous polyposis to confirm the

pharmacokinetics in this population and to measure more detailed pharmacodynamics on repeat biopsy specimens. A subsequent large randomised phase III trial is planned in patients with adenomas detected at screening.

A similar approach to the development of silipide and resveratrol has been adopted by our group. For resveratrol, a single-dose pilot study with detailed pharmacokinetics is nearing completion and will be followed by a repeat dose study in healthy volunteers. Doses between 0.5 and 5 gms daily have been employed and, following analysis of pharmacokinetics and pharmacodynamics measured during this study, a dose will be chosen for future development in the pre-surgical model of patients awaiting colorectal cancer resection. Future phase II and III development with resveratrol will then be undertaken following an analysis of these results.

Silipide is already in phase I development in patients with colon and prostate cancer and incorporates comparison of pharmacodynamics in diagnostic and surgical resection specimens after exposure to this agent.

In summary, therefore, early clinical trials with diet-derived polyphenols are attractive because many of these agents have mechanisms of action which indicate the potential for chemoprevention whilst the extent of experience of long-term exposure in the normal population suggests that they should be well tolerated and safe. Early studies, to date, have confirmed the safety and high level of tolerance of curcumin, resveratrol and silibin and a model of sequential phase I studies with single daily dosing, repeat dosing and use in pre-surgical resection patients provides detailed pharmacokinetic and pharmacodynamic data which allow plans to be made for subsequent phase II and III trials.

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DISCUSSION:
Promise and limitations of current clinical studies – where do we go from here?

Soria

PREVENTION – THERAPY CONVERGENCE AND THE ORAL IEN MODEL

Jon Sudbø

Oral squamous cell carcinoma (OSCC) is a disfiguring, aggressive epithelial malignancy associated with high mortality and severe morbidity in long-term survivors. More than 300,000 annual new cases are diagnosed worldwide. Treating dysplastic oral leukoplakia, the most common oral IEN and a precursor lesion of oral cancer, varies from watchful waiting to complete resection. Oral cancer incidence is increasing, and oropharyngeal cancer is the sixth most common cancer in adults in western countries. Standard screening and local treatment and prior systemic approaches have not reduced the risk or improved the outcome of oral cancer, and so new approaches such as molecular-targeted prevention are needed.

Multifocal, Multistep Carcinogenesis

Oral cancer development is a multifocal process due both to multiclonal development and clonal intraepithelial spread. Multifocality likely is a major cause of the failure of local treatment of oral IEN (particularly aggressive IEN) in preventing oral cancer and supports the testing of systemic therapy with agents targeting signaling pathways relevant to oral carcinogenesis such as the COX-2 and EGFR pathways. The molecular risk markers aneuploidy and LOH are associated with the risks of lateral intraepithelial spread and cancer and are discussed in further detail later in this section under Molecular Risk: Aneuploidy and LOH.

Molecular Risk: Aneuploidy and LOH

Aneuploidy

Over the past 10 years, our group and others have conducted molecular studies of aneuploidy and the loss of heterozygosity for identifying oral IEN patients who have a high risk of oral cancer. Molecular markers of the varying cancer risks of oral IEN have revolutionized drug development in this setting. Substantial evidence points to numerical chromosomal imbalances (aneuploidy) as a cause rather than as a consequence of malignant transformation. Mutations in genes controlling chromosome segregation during mitosis and centrosome abnormalities play a critical role in the development of chromosome instability in cancer. Chromosomal aberrations consistent with impaired fidelity of chromosome segregation during mitosis occur exclusively in aneuploid tumor cell lines. These observations point to a key role of aberrant DNA content in carcinogenesis. Earlier studies of the prognostic potential of DNA quantitations in oral IEN were hampered by limited sample sizes and relatively short follow-up periods (predominantly < 5 years). These limitations have been overcome in more recent studies in Nordic countries mandating the registration and long-term follow-up of dysplastic oral IEN in national tumor registries. These recent studies clearly demonstrated that DNA ploidy analysis—which is a crude quantitation of nuclear DNA content—reliably measures a specific oral IEN's cancer risk. We found that aneuploidy in 45 cases of nondysplastic leukoplakia (11% rate of aneuploidy), 150 cases of dysplastic leukoplakia (17%), and 37 cases of erythroplakia (68%) was a major predictor of cancer development. In the dysplastic IEN patients, cancer developed in 60% with tetraploid and 84% with aneuploid lesions in under 5 years. The risk is even higher in erythroplakia patients with aneuploidy, 92% of whom will develop oral cancer within 5 years. Aneuploidy also marked a high risk of clinically aggressive carcinoma and mortality in leukoplakia or erythroplakia patients. Aneuploidy recently was identified in the scrapings of normal-appearing oral mucosa of 5% to 10% of heavy smokers without IEN, although the risk implications of this

finding are unclear in this non-IEN setting. The reliability of the crude quantitation of DNA content for assessing the cancer risk of oral IEN may seem surprising. Not specific for any particular genetic aberration, DNA ploidy measures global and composite genetic changes emanating from genomic instability, which promotes DNA deletions, amplifications, and other molecular alterations that are critical to oral cancer development. The ploidy data we discuss here are consistent with other oral IEN data indicating that chromosomal polysomy is a major cancer risk factor, and with oral cancer data indicating that aneuploidy is a major predictor of recurrence and poor disease-free and overall survival.

Cancer preventive trials

Therefore, patients with oral IEN and aneuploidy have a very high cancer risk and an urgent need of aggressive prevention interventions. Understanding the mechanism(s) of genomic instability may lead to novel preventive strategies for its suppression, and thus, the prevention or delay of cancer development. Based on showing that aneuploid oral IEN has a >70% risk of becoming biologically aggressive cancer in 3 years that is not improved by complete resection, we have demonstrated that these patients have an unmet medical need calling for a new preventive approach, such as with EGFR and COX-2 inhibition. Complete resection of aneuploid oral IEN does not change cancer rates for these patients (although complete resection may reduce the cancer risk of patients with more-common, lower-risk diploid dysplastic oral IEN). Aneuploidy (and LOH) is associated with multifocal disease (with oral cancers frequently developing in sites distant from the preceding IEN) resulting from lateral spread and likely contributing substantially to the failure of local therapy. Furthermore, the high cancer mortality rate of aneuploid oral IEN is not improved despite aggressive standard therapy of oral cancer developing in these patients.

PRECURSOR LESIONS IN THE ORAL CAVITY AND OROPHARYNX: IDEAL TARGET LESIONS FOR CHEMOPREVENTION

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Recent investigations indicated that squamous cell carcinomas in the oral cavity and oropharynx (OSCC) are preceded by large precursor lesions with dimensions up to 10 cm in diameter. These lesions can macroscopically present as white or red areas in the mucosa, designated leukoplakia or eythroplakia, respectively. The majority of precursor lesions, however, are not visible to the naked eye, but only by histology or genetic markers. Within these large precursor lesions the primary tumors develop. After treatment of the primary tumor, mostly by surgery, the lesions are often left behind and frequently cause secondary tumors (Braakhuis *et al.*, Cancer Res 63:1727, 2003). The realization that the large majority of OSCCs are preceded by these large precursor lesions enables screening and early intervention. A large problem is that only a minority of the lesions is visible by the naked eye, hampering the identification, excision and follow-up monitoring of these lesions. Taking random biopsies for histological examination causes serious discomfort to the patients and is not suited for screening and monitoring. Based on the known patterns of genetic alterations in the precursor lesions preceding OSCCs we have developed two genetic assays that allow non-invasive screening of small brushed samples of the oral cavity. Our data show that particularly loss of heterozygosity genetic analysis with 12 selected microsatellite markers seems a promising screenings assay for non-invasive early diagnosis of precursor lesions in the oral mucosa, with a high analytical sensitivity and specificity. This assay is currently applied on brushed samples of the oral mucosa of leukoplakia patients and a large cohort of controls. These lesions, and the tools that we developed to detect and monitor them, are an excellent opportunity for chemoprevention studies. Currently we are developing immunologically retargeted conditionally replicating adenoviruses to treat these lesions.

BIOMARKERS OF RISK IN PATIENTS WITH ORAL PREMALIGNANT LESIONS

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Oral leukoplakia is a well known premalignant lesion in the oral cavity, defined as a predominantly white lesion of the oral mucosal that cannot be characterized as any other definable lesion. The incidence increases with age with most patients over the age of 50. It develops into oral squamous cell carcinoma (OSCC) in approximately 3% of inflicted patients per year (1). Most oral squamous cell carcinomas, however, are not preceded by leukoplakias, but by non-visible precursor lesions (2). Concerning patients with leukoplakia, the patient related risk factors are female gender and absence of smoking. As a part of the clinical procedure histopathology is done and epithelial dysplasia is graded. In general, a more severe dysplasia is associated with a higher risk of cancer. The practical value of dysplasia grading, however, is questionable, because of the assessment is subjective and inaccurate. Some recent studies focused on the identification of molecular biomarkers associated with OSCC risk. Most studies are retrospective in nature and have a case-control design. Evidence for an increased cancer risk have been found for: increased number suprabasal immuno-stained p53 positive cells (3), level of allelic imbalance (4, 5, 6) and aneuploidy (6, 7). In general, a greater level of genetic instability parallels a higher cancer risk. The results are promising, but before they can be applied in clinical prevention studies, they need to be validated in a prospective setting.

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Cervical Biomarkers in Chemoprevention Trials

Anne-Therese Vlastos

Background

Only exceeded by breast and colorectal cancer, cervical cancer remains the third most common malignancy worldwide (1), despite well known risk factors such as HPV infection (2-4), multiple sexual partners, early age at first sexual intercourse, immunosuppression, smoking, and a good accessibility for cytologic samples (Papanicolaou smear) and histologic analysis with colposcopically directed biopsy.
With a well defined natural history of progression to cancer (5), cervical intraepithelial neoplasia (CIN), also called cervical squamous intraepithelial lesions (SILs), provides one of the best model for various types of research, including chemoprevention trials.

Biomarkers

Biomarkers are used as intermediate indicators of cancer incidence reduction in chemoprevention studies. As defined by Kellof *et al.* (6), a surrogate endpoint biomarker (SEB) must be expressed differentially in normal and high-risk tissue; the marker should appear at a well defined stage of carcinogenesis; the marker must provide acceptable sensitivity, specificity, and accuracy; the marker should be easily measured; the marker should be modulated by chemopreventive agents; and, finally, modulation of the SEB should correlate with a decrease in the cancer incidence rate. Follen *et al*. (7) classified SEBs in six main families: i) Quantitative histopathologic and cytologic markers, ii) proliferation markers, iii) regulation markers, iv) differentiation markers, v) general genomic instability markers, and vi) tissue maintenance markers. As it is impossible to be exhaustive, only some of them will be discussed during the presentation.
All these SEBs are based on cytological or histological samples. New classes of potential novel biomarker of disease progression and regression might be provided by optical technologies.

Future Biomarkers

New technologies provide real-time, *in vivo* information regarding the redox ratio, chromatin distribution, and the nuclear-to-cytoplasmic ratio. Main optical technologies include fluorescence and reflectance spectroscopy, optical coherence tomography (OCT) and confocal imaging in association with optical contrast agents.
OCT is a noninvasive technique able to map subsurface tissue structure with a resolution of 10 to 20 micron. The objective of the Zuluaga *et al*. study (8) was to determine whether an OCT imaging system could be used clinically *in vivo* and they found that image features of normal and abnormal cervical epithelium differ significantly. Carlson *et al.* demonstrated that reflectance and fluorescence confocal microscopy can image cervical epithelium *ex vivo* and *in vivo* with the ability to differentiate between normal and abnormal cervical tissue (9). Once validated, optical biomarkers could help monitor disease regression, persistence, or progression in patients *in vivo* without biopsy.
Optical contrast agents, targeting specific biomarkers associated with increased progression of disease would also provide good SEB. Nida *et al.* published data on quantum dots, an alternative to organic fluorophores for biological imaging, used in conjunction with optical detection methods for imaging. In this study, they directly targeted epidermal growth factor receptors (EGFR) with quantum dots conjugated to anti-EGFR antibodies. Combined with optical imaging technologies, quantum dots helped visualizing changes in cervical cancer at the molecular level (10).

Conclusion

Many SEBs studies are on going in cervical lesions including new optical technologies. Because biopsy itself induces regression, the use of these optical technologies would allow investigators to monitor patients safely throughout clinical trials of new agents. The search for the Perfect SEB is of main interests as it should be suitable to monitor all kind of trials from chemoprevention to vaccines.

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BIOMARKERS TO ASSESS EFFICACY OF BREAST CANCER INTERVENTIONS

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The breast cancer prevention trials are associated with a 40% reduction in risk with tamoxifen and 60% for raloxifene and aromatase inhibitors (AIs) reduce contralateral breast cancer risk by approximately 70%. However, it is estimated that about 50 women need to be treated to prevent one breast cancer. Thus, not only do we need biomarkers for efficacy of preventive agents but better markers of risk than the current ones which are an algorithm of known risk factors. Biomarkers of risk and efficacy may be the same (as with lipid measurements in cardiovascular disease), examples being estrogen, testosterone, SHBG and IGF1. Change in serum concentration of these markers with, for example, oophorectomy, AIs or calorie restriction indicate that risk reduction might occur but not the particular individual who benefits. Similar considerations apply to biomarkers within the breast itself. Mammographic density is associated with a five-fold variation in risk and reduction of density occurs with reductions of estrogen concentrations. It is not known whether the women who have a reduction are the ones who benefit. Cellular atypia in nipple aspirates, ductal lavage specimens and fine needle aspirates (FNAs) is detectable in a proportion of women and on follow up is associated with increase risk of breast cancer. Use of atypia change after an intervention (-difluromethylornithine) lasting 6 months was compared with placebo using multiple FNAs. There was a 28% improvement in cytologic atypia in both groups. Use of atypia as an endpoint requires screening of large numbers in the trial and investigators have turned to other markers of response such as proliferation, ER expression and molecular techniques including gene arrays. Currently the NCI is sponsoring a multi-institutional trial comparing ductal lavage with multiple FNAs before and after 12 months of celocoxib (endpoints – cytomorphology, Ki-67, ER and COX-2) in order to determine the optimal technique. Multiple other studies are underway and some will be reported. Currently the field of biomarkers of risk of breast cancer and for efficacy of interventions is in evolution.

INTEGRATING PROTEOMIC TECHNOLOGIES IN BREAST CANCER PREVENTION RESEARCH

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The completion of the human genome as well as the explosion of novel technologies within genomics, proteomics and functional genomics promise to have a major impact on clinical practice, as these technologies are expected to accelerate the translation of basic discoveries to the clinical practice. In particular, proteomic technologies are expected to play a key role in the study and treatment of cancer, as they provide invaluable resources to define and characterize regulatory and functional networks, investigate the precise molecular defect in diseased tissues and biological fluids, and for developing specific reagents to precisely pinpoint a particular disease or stage of a disease. For drug discovery, proteomics assist with powerful tools for identifying new clinically relevant drug targets, and provide functional insight for drug development.
Today, the application of novel technologies from proteomics and functional genomics to the study of cancer is rapidly shifting to the analysis of clinically relevant samples such as fresh biopsy specimens and biofluids. Being a patient-oriented organisation, The Danish Cancer Society catalysed in 2002 the creation of a multidisciplinary research environment, The DCTB, to fight breast cancer. At DCTB, we have pioneered the application of multiple paradigms from genomics, proteomics and functional genomics to the prospective analysis of fresh tissue samples dissected from patients immediately after surgery, along with the systematic analysis and integration of the corresponding biological and clinical data sets. The direct access to and use of fresh patient material, as well as the close collaboration between basic researchers, surgeons, clinicians and pathologists is one of the unique features of the Centre.
The Centre studies have so far focused on the identification of biomarkers for early detection, as well as for patient stratification for response to treatment, but recently we have managed to develop new approaches to analyse increasingly smaller tissue samples by gel-based proteomics in combination with mass spectrometry and immunohistochemistry, a development that has made possible to undertake a systematic prospective and retrospective analysis of pre-malignant breast lesions with the aim of deriving potential targets for developing novel chemoprevention strategies.

Here I will present the overall strategy of the Centre as well as our ongoing efforts to characterise pre-malignant lesions.

VEGETABLE-INDUCED GENE EXPRESSION CHANGES IN ANTI-CARCINOGENIC PATHWAYS IN HUMAN AND MOUSE COLON

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Abundant epidemiological evidence exists that colon cancer is influenced by environmental factors, with diet as a major determinant. Particularly, vegetable consumption is associated with reduced colon cancer risk. Vegetables contain a wide variety of substances with anti-carcinogenic properties, and some of them may influence gene activities. In order to investigate this, a human dietary intervention study was conducted to identify genes differentially expressed by vegetables *in vivo* in human colon epithelium by microarray technologies. By comparison of pre- and post-intervention values and also the experimental dose groups, genes deregulated by vegetables were identified. Many modulated genes are known to be related to (colon) carcinogenesis. Almost all the effects can be mechanistically linked to cellular processes that explain either prevention of colorectal cancer risk by high vegetable intake or increased colorectal cancer risk by low vegetable intake. In addition, mouse studies were performed, in which for many genes a significant dose-dependent , though not linear, effect was detected. Furthermore, several of these genes were also regulated by one or more of the specific vegetables. Also here, the altered gene expression can indeed explain reduced cancer risk. Few genes, however, are similarly affected in human and mouse.

DISCUSSION:
Promise and limitations of current clinical studies – How realistic is the use of biomarkers to assess study outcome?

ROLE OF THE PHARMACEUTICAL INDUSTRY IN CANCER CHEMOPREVENTIVE AGENT DEVELOPMENT

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Cancer chemoprevention presents a dilemma for the pharmaceutical industry. There are now proven examples of agents which prevent or reduce the risk of cancer. Yet industry remains relatively reluctant to invest significantly in chemoprevention. There are a number of key reasons for this. The first is intellectual property, the second establishing valid endpoints for chemoprevention studies and the third is issues around the safety of pharmaceutical chemopreventives and finally there are issues around subject and physician selection for trials.

Currently the industry has evaluated agents for prevention which have either demonstrated activity in established disease (e.g. tamoxifen) or in other indications (e.g. COX2 inhibitors).This means that a large proportion of the patent life has been taken up and therefore a chemoprevention indication is unlikely to be established before patent expiry thus not allowing the company to make a return on their investment. This has deterred many companies from carrying out chemoprevention studies.

The industry is concerned about relevant endpoints. It could be argued that a reduction in the number of cancers occurring in population is a surrogate from the true endpoint which is a reduction in death rate from that cancer. It has been shown in some trials that the overall reduction in cancer is reduced with intervention, but that the cancers which occur are of a higher grade and worse prognosis. Earlier surrogates are, in the main, difficult to establish and it is hard to convince regulatory authorities of their validity.

Most agents used for the treatment of cancer have a relatively narrow therapeutic index. This is a problem in the prevention setting where even in a selected population of high risk subjects, most do not require the agent. The safety profile of potential chemopreventive agents is studied carefully by regulatory authorities which have stopped a number of trials on the basis of safety concerns.

Finally there are important issues around which subjects should be selected for treatment and the clinical setting in which they will be treated. Whilst most of the research will be carried out by oncology experts, for a chemopreventive to be of maximum value to a population, it must be administered and managed within a primary care setting.

For these reasons it can be seen that chemoprevention represents a unique and difficult challenge for the pharmaceutical industry.

CANCER PREVENTION STUDIES: WHAT CAN WE LEARN FROM SUPPLEMENTS?

Christian Steffen

Dietary factors such as fat and sugar are thought to be responsible for 30 % of all malignancies in Western countries [1]. This diet is associated with an increased proportion of carcinomas of colon, rectum, breast, prostate, and lung.
Attempts to identify single carcinogenic compounds in food have not been successful. Alcohol has been identified as a risk factor in breast carcinoma [2], whereas consumption of fat [3] had no significant influence.
Insufficient evidence impedes the marketing of dietary supplements as prophylactic agents for cancer. One often used argument is the alleged deficit of nutritients in "modern food" that requires supplementation. Others claim that high doses of vitamins and minerals have pharmacologic actions or believe that herbal compounds have protective or curative properties.
For marketing purposes, epidemiologic associations or isolated in-vitro studies are sufficient to claim causal evidence. Consumption of cabbage is associated with low cancer rates [4] and glucosinolates and isothiocyanates with enzyme-inducing properties that protected laboratory animals from carcinogens like dimethylbenzanthracene were isolated from broccoli sprouts [5]. This is sufficient evidence to promote treatment of breast carcinoma with indole-3-carbinol which is formed by enzymatic cleavage of the glucosinolate glucobrassicin.
Other secondary plant compounds such as isoflavones from soy or antioxidant extracts from grape seeds or pine bark are marketed without evidence of efficacy.

The use of high doses of vitamins, especially vitamin C, was propagated by Linus Pauling whose scientific reputation made his "orthomolecular medicine" popular. He recommended the use of vitamin C to treat the common cold as well as colon carcinoma. 10 g vitamin C daily was supposed to increase the average survival by a factor of four, compared to a historic control group [16]. The proof of the inefficacy of this treatment by a prospective study from the Mayo clinic [14] neither impressed promoters or users. Vitamin sales in the USA surpassed 3*109 $ in 1997 and have increased since. In Germany, M. Rath disseminates his abstruse theory that lack of vitamins is the cause of all disease including myocardial infarction and cancer. By using the internet and a sectarian group of users he sells his products by mail from his company seat in the Netherlands. Even German pharmaceutical companies promote vitamin C for tumour therapy without sufficient scientific evidence or the necessary approval from the competent authorities.

Like Vitamin C and E, selenium is promoted as an effective agent for several diseases. The multitude of alleged associations such as sudden infant death [6], HIV [7], breast cancer [8], and secondary lymphedema [9] could not be verified. Contrary to early publications, large epidemiologic studies in Finland [10] and the USA [11] could not find an association between selenium and breast cancer. The indiscriminate use of selenium has led to acute intoxications [12, 13].

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EU PROGRAM PRIORITIES FOR CANCER: FROM FP6 TO FP7

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The Sixth Framework Programme (FP6) of the European Union runs from 2002 to 2006 and focuses on the creation of a lasting and coherent research structure in Europe, the European Research Area. With an overall budget of 17.5 billion euro, FP6 is structured around three main pillars:

1) Focussing and integrating research in the European Union
2) Structuring of the European Research Area
3) Strengthening the foundations of the European Research Area

Seven thematic priorities have been established under the first of the pillars. Cancer research is an important research field within one of these: 'Life Sciences, genomics and biotechnology for health'. During FP6's 4-year lifespan, a total of approximately 450 million euro will be dedicated to cancer-related projects carried out by international and interdisciplinary consortia.

The underlying objectives of the cancer research funded by the European Commission are to develop improved patient-oriented strategies, from prevention to more effective and earlier diagnosis and better treatment with minimal side effects. The research will therefore concentrate on translating the knowledge being created by genomics and other fields of basic research into applications that improve clinical practice and public health.

In total, four FP6 calls for proposals will be carried out in the Life Sciences, three of which have been closed by now. Two topics offered through the calls for proposals were directly relevant for cancer prevention/chemoprevention, and 6 out of the 43 projects funded through the first two cancer calls have research elements focused on prevention/chemoprevention.

A proposal for the Seventh Framework Programme (FP7), which will run from 2007 to 2013, has been presented by the European Commission on 6.4.2005 (COM(2005) 119). It is build on continuity, but also contains some innovative elements, such as the European Research Council for basic research. An ambitious doubling of the budget for FP7 has been proposed, which should – if implemented - also lead to doubled budgets for health research. Cancer will be funded as before through calls for proposals within health research.

The proposal has been presented simultaneously to the European Parliament and the Council, where it is currently discussed.

A detailed description of cancer research funding under FP6 as well as a summary of the first, second and third call for cancer-related topics with a focus on prevention/chemoprevention will be given and the current state of affairs of FP7 discussed.

DISCUSSION:

The European dimension: How can we improve clinical cancer chemopreventive agent development in Europe?