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DIRECCIÓN DE NUEVAS CREACIONES

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Abogada
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ASUNTO Radicación 10 117 653
 Trámite 011
 Evento 378
 Actuación 519
 Oficio 15
 Folios 15 - 196 7 7

Patente de Invención	☒	Patente de Modelo de Utilidad	☐
Vía Nacional	☐		
Vía PCT (Fase Nacional)	☒	Capítulo I	☒
		Capítulo II	☐
No. Solicitud Internacional	PCT/JP2009/054007		
Fecha de Presentación Internacional	25/Febrero/2009		

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 763 a 1396	23/Septiembre/2010
	Folios 1419 a 2048	30/Junio/2011
Reivindicaciones:	Folios 1397 a 1400	23/Septiembre/2010
	Folios 2049 a 2052	30/Junio/2011
Secuencias:	Folios 1402 a 1405	23/Septiembre/2010
Prioridad:	JP 2008-045134	26/Febrero/2008
	JP 2008-256755	01/Octubre/2008

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

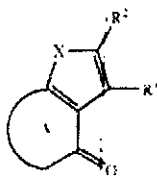
3. Objeto de la invención

Título de la solicitud: "Derivado Heterocíclico Fusionado Que Tiene Una Actitud Inhibidora de SMO y Baja Toxicidad" (modificado en el folio 1415).

El problema planteado en esta solicitud consiste en la necesidad de proporcionar un compuesto que tiene actividad inhibidora de Smo superior, baja toxicidad y es suficientemente satisfactorio como un producto farmacéutico útil como agente para la profilaxis o el tratamiento del cáncer, un inhibidor del crecimiento del cáncer y un

agente supresor de metástasis del cáncer.

Para resolver este problema técnico la solicitud en estudio proporciona un compuesto de la fórmula (I) y composiciones que lo contienen:



El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C07D 471/004.

4. Reivindicaciones relativas a un uso ó a un segundo uso (Art 14 y 21 D 486)

No obstante la reivindicación 15 se encabeza como un agente farmacéutico, éste se define en términos de su uso como agente para la profilaxis o el tratamiento del cáncer y por lo tanto no es patentable, de acuerdo con el Art 14.

5. De la Solicitud de Patente

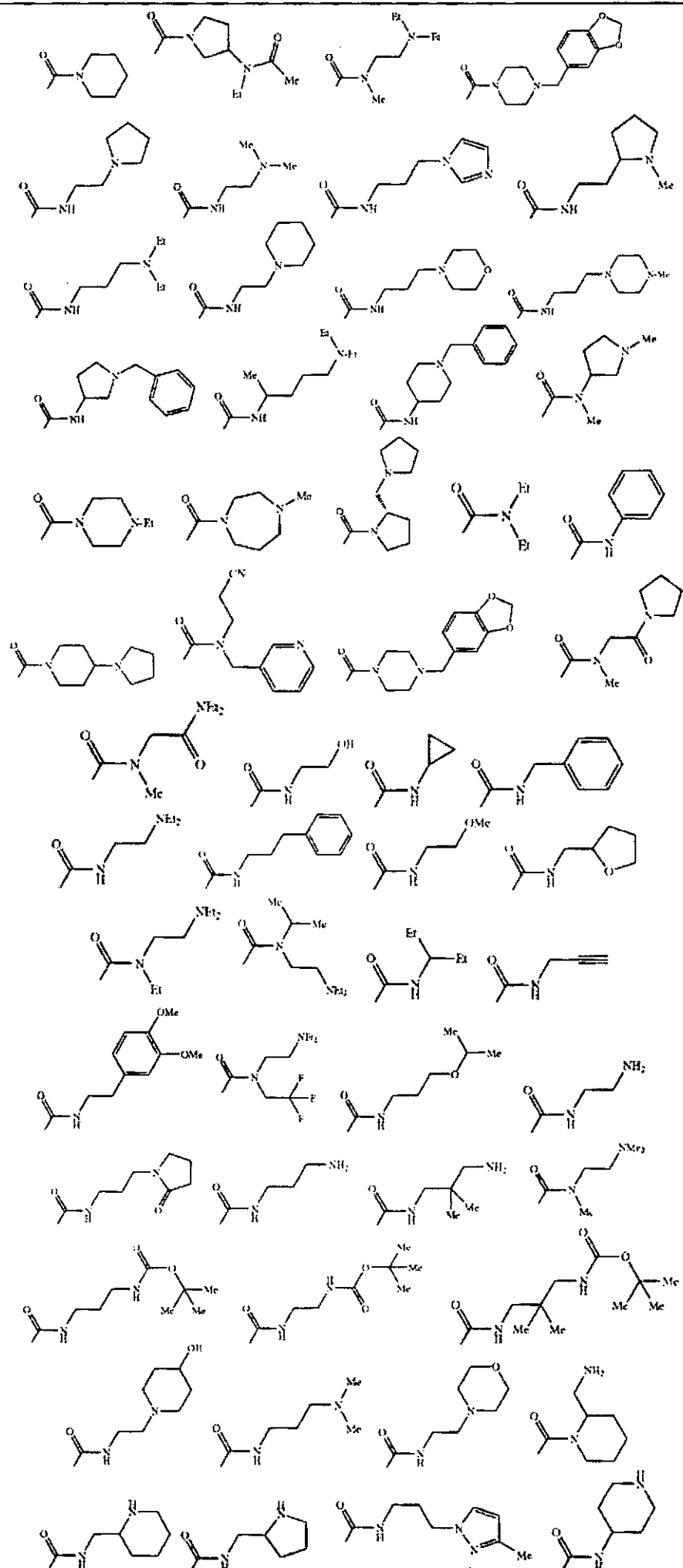
Título

No es claro a que se refiere el solicitante con "actitud" en el título de la solicitud. Se sugiere al solicitante las aclaraciones necesarias y hace el título más concreto según las características estructurales del compuesto objeto de la solicitud.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones 1 – 6 no son concisas porque las definiciones de A, B, X, R², R³, R^{AA1}, R^{AA2}, R⁴, R^{A1} y R^{A2} nombran que son opcionalmente sustituidos, sin definir cuales son los posibles sustituyentes en cada uno de los casos y la definición de R¹ incluye un "grupo hidrocarburo", sin definir a cual grupo se refiere. Esta forma de definir no es adecuada porque da lugar a un sinnúmero de compuestos que no encuentran soporte en la descripción, por lo tanto se sugiere restringir la solicitud a una razonable generalización del tipo de compuestos que ha sido sintetizado ó probado, es decir, que se encuentran debidamente soportado. Se aclara al solicitante que en la definición es necesario incluir los radicales específicos con sus átomos, números de átomos y anillos heterocíclicos específicos con el fin de definir la materia de forma inequívoca. A continuación se encuentran algunos ejemplos para ilustración:

Sustituciones	Soporte de los ejemplos presentados

R²

R ³	-OCH₃, -O(CH₂)₂OCH₂CH₃, -OOCH₂CH₃, -O(CH₂)₂CH₃, -OCH₂CF₃, -OCH₂CH₂F, -O(CH₂)₃CH₃
R ⁴	H, -CH₃, -(CH₂)₃CH₃, -CO₂H,

Las Reivindicaciones 7 – 11 se encuentran como independientes, sin embargo, éstas no se refieren a materia diferente de la reclamada en la reivindicación principal 1, por

lo tanto se sugiere hacerlas dependientes de alguna de las reivindicaciones anteriores.

Las reivindicaciones 12 y 13 se refieren a un profármaco del compuesto de la solicitud, sin definirlo por sus características técnicas esenciales, adicionalmente, no se encuentra soporte descriptivo de ningún profármaco. En consecuencia, se sugiere eliminar esta reivindicación del alcance del pliego reivindicatorio.

No es claro a que se refiere el solicitante con agente farmacéutico en las reivindicaciones 13 y 14, éstas no se encuentran definidas por características técnicas y por lo tanto no es posible distinguirlas del estado de la técnica. Además, la reivindicación 13 se esta definiendo por la actividad como inhibidor de Smo, que es un resultado a alcanzar, no define al producto, puesto que la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se sugiere reemplazarlo por las características esenciales, estructurales o funcionales, que contribuyen a la obtención de ese efecto.

6. Unidad de invención (Art 25 D 486)

La Solicitud no cumple con el requisito de unidad de invención, porque en el documento D1 ya se divulgaban compuestos de fórmula (I) inhibidores del efecto de radicales libres y por lo tanto útiles para el tratamiento y profilaxis de cáncer. En consecuencia la solicitud se refiere a 2 grupos inventivos.

Los grupos inventivos están definidos por las siguientes características **esenciales**:

- 1° Compuestos de fórmula (I'') (Documento D1)
- 2° Compuestos de fórmula (I')

Se sugiere presentar las solicitudes divisionales que considere necesarias, ó limitar la solicitud inicial a una sola invención, eliminando de ella las otras invenciones.

7. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: Febrero 26 de 2008 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	US 5,360,799	Substituted Thienyl or Pyrrolylcarboxylic Acid Derivatives, Their Preparation and Medicines Containing Them	01/Noviembre/1994

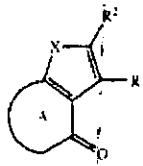
D1 http://worldwide.espacenet.com/publicationDetails/originalDocument?FT=D&date=19941101&DB=EPODOC&locale=en_EP&CC=US&NR=5360799A&KC=A&ND=2 divulga (Col. 1, línea 13 – Col. 2, línea 12) un compuesto de la fórmula (I) inhibidores del efecto de radicales libres oxigenados biológicos, que son la fuente de muchas enfermedades degenerativas desde enfermedades inflamatorias o autoinmunes hasta la insuficiencia respiratoria y cardiaca, o isquemias intestinal o cerebral, también se encuentran implicados en el fenómeno degenerativo del envejecimiento.

8. Examen de Patentabilidad (Art 14 D486)

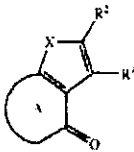
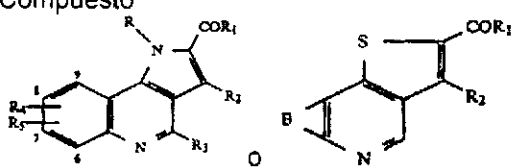
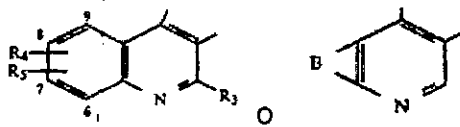
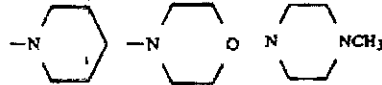
Nivel Inventivo (Art18 D486)

GRUPO INVENTIVO 1

La reivindicación 1 consiste en un compuesto caracterizado por la fórmula (I) o una sal del mismo.



Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1
Un compuesto de fórmula (I)  o una sal del mismo.	Compuesto 
A : un anillo de 5 a 6 miembros que esta opcionalmente sustituido, donde los sustituyentes están unidos opcionalmente entre sí para formar un anillo	
X : O, S o NR ¹ R¹ : H o un grupo hidrocarburo que esta opcionalmente sustituido	S, N
R² : carbamoilo opcionalmente sustituido	-COR ₁ R ₁ : -N(C ₃ H ₇) ₂ , -N(C ₅ H ₁₁) ₂ , -NH ₂ 
R³ : hidroxí opcionalmente sustituido	-OH, -OC ₂ H ₅ ,

El documento D1 revela un compuesto de la fórmula (I) inhibidor del efecto de radicales libres oxigenados biológicos, los compuestos del documento D1 coinciden con los compuestos de fórmula (I) de la solicitud cuando A es un anillo de 5 a 6 miembros que está sustituido, donde los sustituyentes están unidos opcionalmente entre si para formar un anillo, X es S o N, R² es carbamoilo opcionalmente sustituido y R³ es hidroxí opcionalmente sustituido (Columnas 12 – 25, Tablas 1 – 4)

La diferencia entre la invención, definida en la reivindicación 1 y el compuesto del documento D1 consiste en el carbonilo.

El efecto que logra el incluir el carbonilo es la obtención de compuestos inhibidores de Smo útiles para el tratamiento de cáncer.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo modificar los compuestos conocidos en el documento D1 para obtener compuestos alternativos para el tratamiento de cáncer”.

Sin embargo, los compuestos de la solicitud solo difieren de los compuestos del documento D1 en el carbonilo, y esta anterioridad ya divulgaba la actividad inhibitoria del efecto de radicales libres, que son fuente de muchas enfermedades degenerativas desde enfermedades inflamatorias o autoinmunes hasta la insuficiencia respiratoria y cardíaca, o isquemias intestinal o cerebral y que también
Expediente 10 117 653 1er Art 45

se encuentran implicados en el fenómeno degenerativo del envejecimiento (Col. 1, líneas 13 – 26); y es conocido por la persona normalmente versada en la materia que los radicales libres están relacionados con la inflamación crónica y el cáncer ⁽¹⁾. Por lo tanto el hecho de identificar que los compuestos tienen acción inhibitoria de Som no le otorga nivel inventivo a los compuestos de la solicitud.

En consecuencia, la persona del oficio normalmente versada en la materia, realizaría pequeñas modificaciones como la inclusión de un grupo carbonilo sobre los compuestos del documento D1 que tienen actividad inhibitoria del efecto de radicales libres, los cuales se encuentran implicados en el desarrollo de patologías como el cáncer con una amplia expectativa de mantener la actividad, llegando así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

Puesto que las reivindicaciones dependientes 1 - 8 y 12 - 14 no mencionan características inventivas adicionales tampoco se pueden considerar inventivas.

Conclusión:
Las reivindicaciones 1 - 8 y 12 - 14 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

Conclusión:
Las reivindicaciones 1 – 8 y 12 - 14 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

9. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	US 5,360,799	1 – 8 y 12 - 14	Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.


JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha 30 OCT. 2012

Elaborado por: Jeny Paola Villate Becerra *P. Villate*
Revisado por: Consuelo Leguizamón *[Signature]*

(1) HUSSAIN, S. ET AL, "Radical Causes of Cancer", Nature Reviews, Cancer, Vol. 3, No. 4, Págs. 276 – 285.

RADICAL CAUSES OF CANCER

S. Perwez Hussain*, Lorne J. Hofseth* and Curtis C. Harris

Free radicals are ubiquitous in our body and are generated by normal physiological processes, including aerobic metabolism and inflammatory responses, to eliminate invading pathogenic microorganisms. Because free radicals can also inflict cellular damage, several defences have evolved both to protect our cells from radicals — such as antioxidant scavengers and enzymes — and to repair DNA damage. Understanding the association between chronic inflammation and cancer provides insights into the molecular mechanisms involved. In particular, we highlight the interaction between nitric oxide and p53 as a crucial pathway in inflammatory-mediated carcinogenesis.

CYTOKINE

A soluble protein that is produced and released by individual cells that transmit distinct messages of activation, inhibition, chemoattraction or apoptosis to other cells. This interaction triggers effector mechanisms within the responding cell. Key pro-inflammatory cytokines include IL-1 β , TNF- α and IFN- γ .

FREE RADICAL

An atom or a group of atoms that has an unpaired electron. These are highly reactive to biological molecules and will damage them.

The infectious and non-infectious generation of chronic injury and irritation initiates an inflammatory response^{1,2}. 'Go' signals² — such as bioactive peptides from neurons, CYTOKINES or receptor molecules that sense microbes — lead to the recruitment of mast cells and leukocytes to the damage site. A subsequent 'respiratory burst' — an increased uptake of oxygen that leads to the release of FREE RADICALS from leukocytes, including activated macrophages — can damage otherwise healthy neighbouring epithelial and stromal cells. This process can drive carcinogenesis by altering targets and pathways that are crucial to normal tissue homeostasis (reviewed in REFS 1,3). It has been estimated that chronic infection and associated inflammation contribute to about one in four of all cancer cases worldwide⁴.

Targets of free radicals in inflammation include DNA, proteins, RNA and lipids (FIG. 1). Mutations in cancer-related genes or post-translational modifications of proteins by NITRATION, NITROSATION, phosphorylation, acetylation or polyADP-ribosylation — by free radicals or LIPID PEROXIDATION byproducts, such as the reactive aldehydes MALONDIALDEHYDE and 4-hydroxynonenal — are some of the key events that can increase the cancer risk. In addition, free radicals can modulate cell growth and tumour promotion by activating signal-transduction pathways, which results in the transcriptional induction of proto-oncogenes, including c-FOS, c-JUN and c-MYC⁵, that are involved in stimulating growth. Mechanistic studies have shown that protein phosphorylation and polyADP-ribosylation of chromosomal proteins are

Involved in the transcriptional induction of c-FOS by oxidants⁶ and that a pro-oxidant state can promote neoplastic growth⁷.

TABLE 1 summarizes examples of oxyradical overload diseases. These develop from conditions of chronic inflammation and can have an aetiology that is primarily inherited or acquired through viral, bacterial and parasitic infection, or acquired through chemical induction. Cancer proneness is frequently a pathological consequence of extensive and sustained free-radical stress-related damage in these diseases. However, the exact mechanisms still require further elucidation. What are the roles of free radicals in cancer proneness in chronic inflammatory — oxyradical overload — diseases? What are the targets of these free radicals *in vitro* and *in vivo*? And, because the tumour suppressor p53 is a crucial target for alteration, how is this molecule involved in the genesis of cancer during chronic inflammation?

Free radicals damage DNA and modify proteins

The hypothesis that free radicals have a role in carcinogenesis comes from *in vitro* studies describing their role in DNA damage, and protein structural and functional modifications. One of the earliest convincing studies showing that free radicals damage DNA came from the observation that hydrogen peroxide (H₂O₂), in the presence of a peroxidation activator, Fe₂(SO₄)₃, induced chromosome fragmentation⁸. Since then, many others have shown an association of other free radicals with DNA damage and protein modifications^{9–13}.

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*These authors contributed equally to this paper. Correspondence to C.C.H. e-mail: Curtis_Harris@nih.gov doi:10.1038/nrc1046

NITRATION

Addition of an equivalent of NO_2^+ in a free-radical mechanism often resulting in the formation of 3-nitrotyrosine. This modification can affect the function of certain proteins and is involved in disease pathology, including cancer.

NITROSATION

Addition of an equivalent of NO^+ (nitrosonium ion or nitrosyl cation) to an amine, thiol or hydroxyaromatic group.

LIPID PEROXIDATION

Auto-oxidation of lipids that are exposed to oxygen.

Summary

- Chronic inflammation deregulates cellular homeostasis and can drive carcinogenesis.
- Free radicals and aldehydes — produced during chronic inflammation — can induce a number of alterations, including gene mutations and post-translational modifications of key cancer-related proteins. These alterations can lead to the disruption of cellular processes such as DNA repair, cell-cycle checkpoints and apoptosis.
- The ultimate effect of free radicals is complex and depends on their local concentration, the microenvironment and the genetic background of the individual.
- Nitric oxide and its derivatives damage DNA and modify protein structure and function but can also protect from cytotoxicity. These 'two faces' of nitric oxide highlight the need for further study before considering nitric oxide as a target for chemoprevention in high cancer risk, chronic inflammatory diseases.
- People with cancer-prone inflammatory diseases, such as ulcerative colitis, haemochromatosis and viral hepatitis, have alterations in cancer-related genes and proteins, which are associated with free-radical stress.
- Transgenic and knockout animal models support the role of free radicals in carcinogenesis.
- Prospective chemoprevention studies are needed to evaluate the use of antioxidants and inhibitors of pro-oxidant enzymes for the prevention of cancer in people with oxyradical overload diseases.

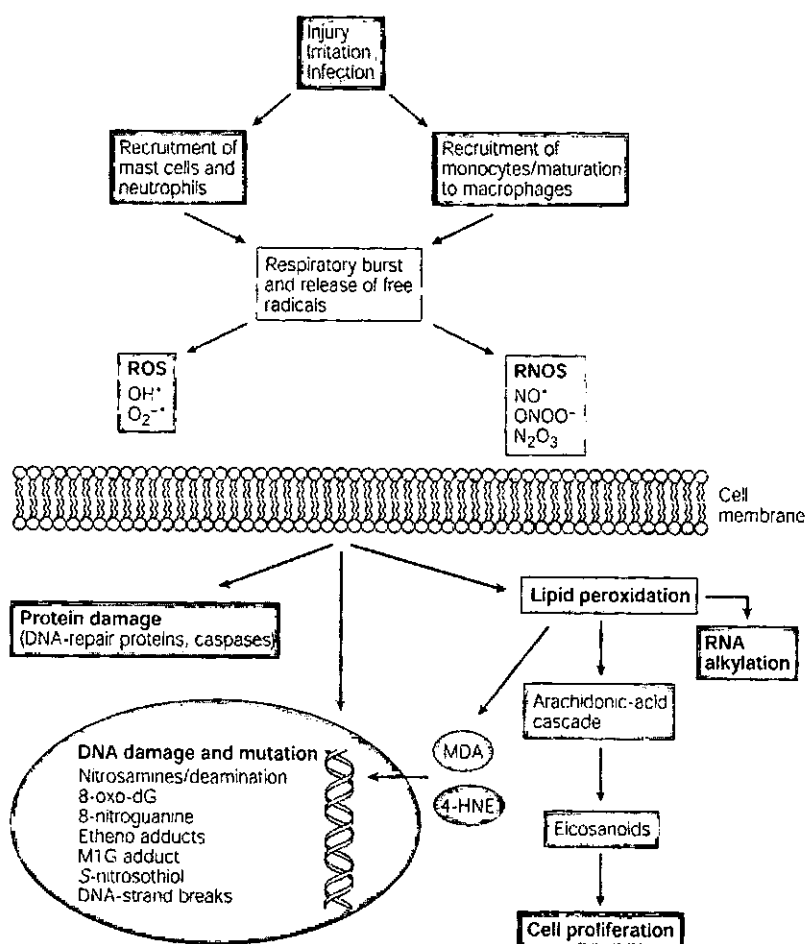


Figure 1 | Impact of free radicals released at sites of inflammation on cellular molecules. Inflammation begins with a reaction to an irritant or infection that is characterized by movement of fluid and white blood cells into extravascular tissue. This is followed by tissue repair and regeneration and involves cell proliferation. Associated with these processes are the release of free radicals, such as reactive oxide species (ROS) and nitrogen oxide reactive species (RNOS). This can activate a process called lipid peroxidation and the arachidonic-acid cascade, with the production of cell-proliferation-stimulating eicosanoids. Also, DNA-damaging agents, such as malondialdehyde (MDA) and 4-hydroxynonenol (4-HNE), are by-products of the arachidonic-acid cascade. The free radicals can also damage DNA and modify the structure and function of cancer-related proteins directly. $\text{OH}^\bullet$, hydroxyl radical; $\text{O}_2^{\bullet-}$, superoxide; $\text{NO}^\bullet$, nitric oxide; $\text{ONOO}^\bullet$, peroxynitrite; N_2O_3 , nitrous anhydride.

Due to the complex chemical and biological nature of free radicals, we are only now beginning to understand the key players involved in inflammatory-mediated carcinogenesis and their specific actions. In this review, we focus on the effects of free radicals on biomolecules in relation to carcinogenesis. The complex chemistry has been extensively reviewed elsewhere^{3,14–16}. Highly reactive molecules to DNA *in vitro* include hydroxyl radicals ($\text{OH}^\bullet$), which cause oxidative DNA damage, and peroxynitrite ($\text{ONOO}^\bullet$), which causes both oxidative damage and nitration of DNA bases. *In vivo*, the relevance of $\text{OH}^\bullet$ to DNA damage, however, remains unclear because it has a very short half-life, and, therefore, has to be produced directly adjacent to DNA to induce damage¹⁷. By contrast, $\text{ONOO}^\bullet$ can diffuse within cells¹⁸, and so might be of particular concern during chronic inflammation. A less reactive molecule — such as nitric oxide ($\text{NO}^\bullet$) — has the ability to diffuse over several cell diameters¹⁹ and might also be relevant. Reactive species that are derived from $\text{NO}^\bullet$ are released from inflammatory cells and can act on neighbouring dividing epithelial cells, leading to somatic mutations in crucial cancer-causing genes.

Test-tube and cell-culture studies have shown that DNA is not the only molecule at risk. Proteins such as DNA-repair enzymes, those involved in signal transduction, apoptotic modulators and the p53 protein can be modified — both structurally and functionally — when exposed to free radicals¹⁰. Key post-translational modifications following free-radical exposure and their functional consequences have been reviewed elsewhere^{20–22}. We²³ and others²⁴ have shown that, after exposure to $\text{NO}^\bullet$ and its derivatives, p53 is post-translationally modified at residues that are crucial to its multiple functions. Others have shown that DNA-repair and signal-transduction molecules, such as DNA-protein kinases, are activated by exposure to $\text{NO}^\bullet$ (REFS 25,26). As a modifier of proteins that are crucial to cell function, $\text{NO}^\bullet$ can influence downstream events in carcinogenesis, including cell-cycle checkpoints²⁷, apoptosis²⁸ and DNA repair^{29,30}. Elucidation of these influences has provided a better

REVIEWS

Table 1 | Examples of high cancer risk, oxyradical overload diseases

Disease	Cancer	Risk*	References
Inherited			
Haemochromatosis	Liver	219	97
Crohn's disease	Colon	3	153
Ulcerative colitis	Colon	6	154
Acquired: viral			
Viral hepatitis B	Liver	88	155
Viral hepatitis C	Liver	30	155
Human papillomavirus infection	Cervix	16	156
Acquired: bacterial			
<i>Helicobacter pylori</i> infection	Gastric	10	157
Urinary bladder catheterization	Bladder	5–28	158
Prostatitis	Prostate	2	179
Acquired: parasitic			
<i>Schistosoma hematobium</i>	Bladder	2–14	134
<i>Schistosoma japonicum</i>	Colon	1.2–6.0	134
Acquired: chemical/physical			
Barrett's oesophagus	Oesophageal	50–100	159
Pancreatitis	Pancreatic	2–3	160

*Relative risk or odds ratio

understanding of how a micro-environment of chronic inflammation can transform a normal cell into a cancer cell (FIGS 1 and 2).

The two faces of nitric oxide

Since its discovery, it has been difficult to identify the specific roles of NO^{*} in carcinogenesis. This is because the effects of NO^{*} are dependent on its concentration, its interaction with other free radicals, metal ions and proteins, and the cell type and the genetic background that it targets. It is fairly clear that NO^{*} mediates angiogenesis^{31–33}. However, NO^{*} can both cause DNA damage and protect from cytotoxicity, it can inhibit and stimulate cell proliferation, and it can be both pro- and anti-apoptotic^{34–46}. It is therefore important to be aware of the conditions used when interpreting results from cell-culture experiments⁴⁷. Depending on the conditions used, DNA-damaging NO^{*} byproducts — such as nitrosoperoxycarbonate, ONOO⁻ and nitrogen dioxide — are often formed^{48–51}. This complicated chemistry and the biological actions of NO^{*} should also be considered when interpreting the results of animal studies^{33,52}.

Inflammation, nitric oxide and p53

Alterations in DNA occur after exposure to high levels of NO^{*} and its derivatives^{53–55}. p53 mediates the response to various stress signals, and exposure of NO^{*} causes p53 accumulation and post-translational modifications²³ that inhibit cellular growth. This can lead to selective clonal expansion of p53-mutant cells following NO^{*} exposure (FIG. 2). We have tested this hypothesis by genetically engineering human cancer cells *in vitro* to produce concentrations of NO^{*} that are similar to those in human cancer. We observed an

increase in the expression of the G1–S cell-cycle checkpoint protein, WAF1, in cells with wild-type p53, and also expression of the enzyme inducible NO synthase (iNOS), which catalyses the production of NO^{*}, leading to reduced tumour growth and increased tumour necrosis as xenografts in athymic nude mice. By contrast, engineered cells with mutated p53 showed an accelerated tumour growth and an increase in the expression of vascular endothelial growth factor (VEGF) and neovascularization⁵⁶. Regulation of VEGF by NO^{*} has been confirmed by other investigators⁵⁷. Furthermore, wild-type p53-induced transrepression of iNOS, as shown in both *in vitro* and *in vivo* conditions^{58,59}, provides a protective mechanism against prolonged exposure to pathological concentrations of NO^{*}. These studies indicate that exposure of cells to a high level of NO^{*} and its derivatives during chronic inflammation in the absence of wild-type p53 — and therefore the negative iNOS regulation — might increase the susceptibility to cancer.

There is also evidence of an association between NO^{*} and its derivatives, and p53 mutations^{60,61}. Both an increase in p53 mutations at codons 247 and 248, and an increase in iNOS expression, are found in inflamed lesions of the colon of patients with ulcerative colitis (UC)⁶¹. Similarly, a high expression of iNOS along with G:C to T:A transversions at codon 249 of p53 is reported in the tissue from patients with haemochromatosis⁶². In colon tumours, iNOS activity is highest in adenomas, then decreases with advancing tumour stage and is lowest in metastatic tumours^{60,63}. One explanation for this reduction in iNOS is the killing of tumour-associated mononuclear cells, which produce iNOS, by Fas ligands in advanced tumours⁶⁴. Interestingly, in colon tumours there is a positive correlation between iNOS activity and G:C to A:T mutations at 5-methylcytosine sites in p53, but the rates of all other mutations vary inversely with iNOS activity⁶⁰. There is also an association between increased iNOS expression and G:C to A:T transition mutations in p53 in stomach, brain and breast cancers^{65–70}. NO^{*} and its derivatives are, therefore, capable of causing mutations in cancer-related genes, inducing clonal expansion of mutated or aberrant cells and promoting angiogenesis — NO^{*} can therefore act as both an endogenous initiator and a promoter in human carcinogenesis.

Animal models of inflammation

Animal models have been developed to provide a better understanding of the mechanisms that are involved in chronic inflammatory-associated carcinogenesis. There are several models for many of the diseases. The first studies — published in the 1940s — described an increased cancer incidence in areas of the body undergoing chemical or physical irritation^{71,72}. Since then, there have been many animal models developed that use irritants to induce chronic inflammation. Often, if the inflammation continues over a prolonged period, cancer develops at the site of

MALONDIALDEHYDE

A naturally occurring, bioactive by-product of lipid peroxidation and prostaglandin synthesis that has the potential to damage DNA. It reacts with DNA to form adducts to deoxyguanosine and deoxyadenosine.

FAS LIGAND

A 40-kDa transmembrane protein that belongs to the tumour necrosis factor (TNF) family. It is a potent pro-apoptotic factor.

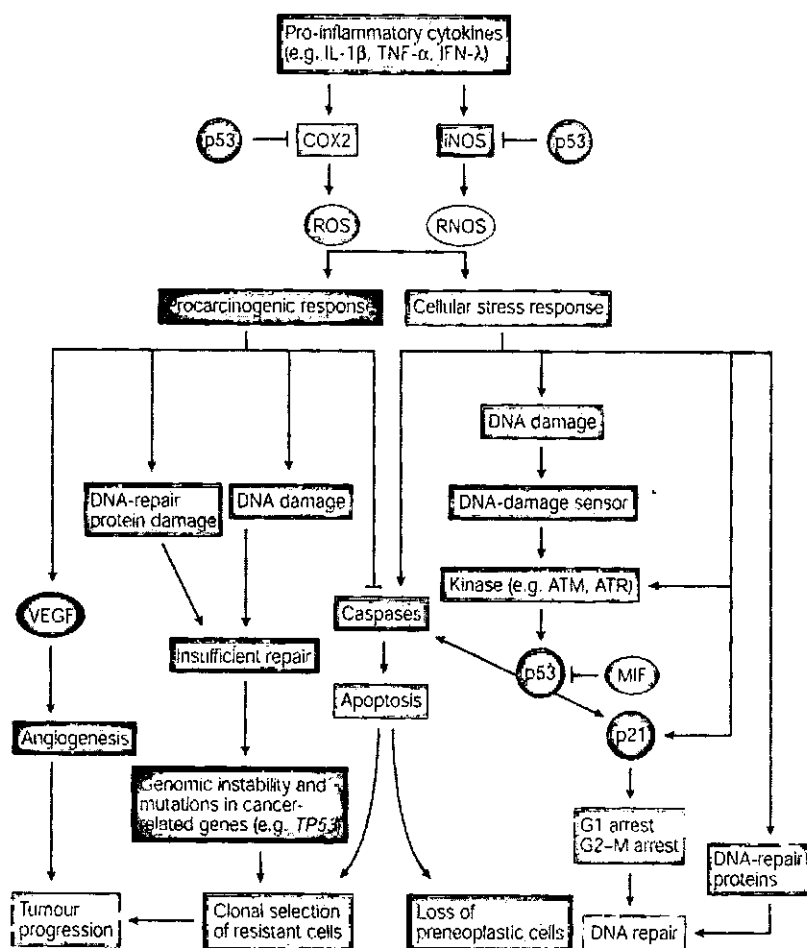


Figure 2 | Free-radical generation, cellular stress and tumorigenesis. Free radicals might inhibit or exacerbate tumorigenesis, depending on concentrations, the cell type, genetic background and the microenvironment in which free radicals act. Pro-inflammatory cytokines induce inducible nitric oxide synthase (iNOS) and COX2 that generate free radicals (for example, reactive oxygen species (ROS) and NO[•]). Both iNOS and COX2 can be transcriptionally repressed by p53 (REFS 56,152). The cellular stress response involves identification of the DNA damage, DNA repair and loss of preneoplastic cells. By contrast, insufficient repair of DNA damage causes a procarcinogenic response, triggering selection of cancerous cells and angiogenesis. ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and RAD3 related; MIF, macrophage inhibitory factor; VEGF, vascular endothelial growth factor.

APC GENE

The adenomatous polyposis coli gene is a tumour suppressor. Mutations in the gene are responsible for familial adenomatous polyposis and most sporadic colorectal cancers. The best understood function of APC is the destabilization of β-catenin, a key effector of the WNT signalling pathway

Irritation and inflammation. One example of such a model is mice that have been fed dextran sodium sulphate (DSS), which leads to the onset of inflammatory bowel disease and, if continued, colon cancer^{73,74}. In another model, surgically opening the duodenum to the gastroesophageal junction — oesophagogastrroduodenal anastomosis — which introduces mixed reflux of gastric and duodenal contents in rats, causes the onset of Barrett's oesophagus and, if continued, oesophageal cancer^{75,76}. In addition, transgenic mice overexpressing the hepatitis B virus large envelope polypeptide develop inflammation and regenerative hyperplasia, and, if continued, liver cancer⁷⁷. *Helicobacter pylori* infection in mice induces chronic inflammation and gastric atrophy, and, if continued, gastric adenocarcinoma⁷⁸.

Knockout models targeting key molecules involved in inflammation and inflammatory-mediated carcinogenesis also provide a better understanding of potential targets for chemoprevention.

IL-10-knockout mice. Interleukin-10 (IL-10) is an anti-inflammatory cytokine and a pleiotropic molecule with diverse effects, including the regulation of T-cell responses, the regulation of acute inflammatory responses and the regulation of free-radical release (reviewed in REF 79). Knocking out the gene for IL-10 in mice leads to enterocolitis⁸⁰, which is similar to human inflammatory bowel disease. This is, therefore, a useful model to study UC and Crohn's disease associated with carcinogenesis. *IL-10^{-/-}* mice have progressive inflammatory changes in the colon and a high incidence of colo-rectal adenocarcinomas⁸¹. The demonstration that the enterocolitis pathology in *IL-10^{-/-}* mice can be partially improved by exogenous IL-10 administration provides additional evidence that this molecule has an important role in inflammation⁸¹. However, the story is complicated by an interesting observation — *Helicobacter hepaticus* has also been shown to be involved in the development of enterocolitis in *IL-10^{-/-}* mice⁸².

iNOS-knockout mice. iNOS can produce micromolar quantities of NO[•], and is a key molecule involved in inflammatory-mediated carcinogenesis⁸³. *iNOS^{-/-}* mice were first generated in 1995 to test the hypothesis that iNOS defends the host against infectious agents and tumour cells at the risk of contributing to tissue damage and shock⁸³. Since then, these mice have proved useful in investigating the role of iNOS in many other pathologies, including inflammation, liver regeneration, tumorigenesis and sepsis-induced hypotension (reviewed in REF 84).

Studies have shown that *iNOS^{-/-}* mice treated with the irritant trinitrobenzene develop early-phase inflammation compared with wild-type mice⁸⁵. Others have found that genetic ablation of the *iNOS* gene confers significant resistance to trinitrobenzene-induced lethality and colonic damage, and reduces the post-translational modification of proteins, such as nitrotyrosine formation and the production of lipid peroxidation byproducts, such as malondialdehyde⁸⁶. Also, if the *iNOS^{-/-}* mice are fed DSS they show reduced signs and symptoms of colitis compared with wild-type mice, indicating that iNOS might have a crucial role in the pathology of colitis⁷⁴. The implications of reduced inflammation of the colon in these mice remains to be determined. An interesting study by Konopka *et al.*⁸⁷ indicated that *iNOS^{-/-}* mice inoculated with B16-F1 melanoma cells develop less tumours, which are associated with lower VEGF expression, than those in wild-type mice, further indicating a role for iNOS in tumour progression⁸⁶. Another recent study found that when mice with a germline mutation in the *apc* tumour-suppressor gene (Min mice) were fed with DSS, they had significantly accelerated colitis, dysplasia and cancer development compared with wild-type mice, indicating

REVIEWS

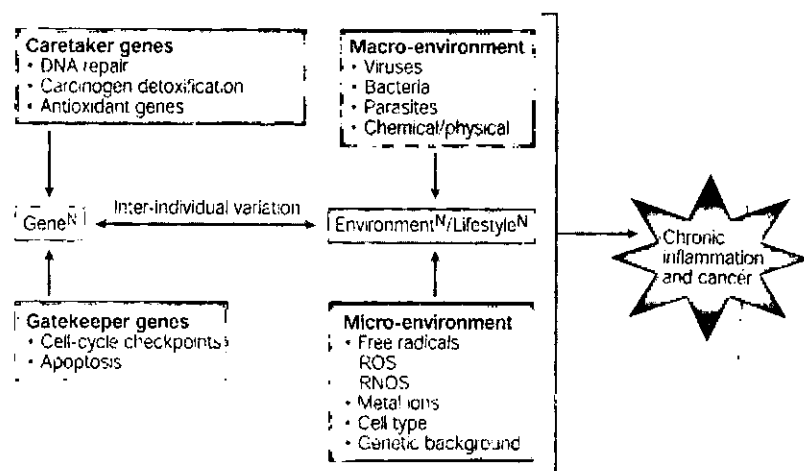


Figure 3 | Gene-microenvironment interactions in chronic inflammation. Genes and environmental exposures contribute to the carcinogenic process in chronic inflammatory diseases. The effects can be additive or multiplicative, and are modifiable by inter-individual variation in genetic function. We propose including antioxidant and base-excision DNA-repair genes as caretaker genes involved in maintaining genomic integrity. N, number; ROS, reactive oxygen species; RNOS, reactive nitrogen oxide species.

that this mutation is important in inflammatory bowel-disease-associated cancer⁷⁷. Finally, Ahn and Ohshima have shown a significant reduction in adenomas in *Min/INOS^{-/-}* mice compared with *Min* mice alone⁸⁸, indicating that NO[•] and its derivatives have an important role in promoting colon carcinogenesis in a background of *APC* mutations.

Cox1- and Cox2-knockout mice. Cyclooxygenases catalyse the transformation of arachidonic acid into prostaglandin H₂, as the first step in the biosynthesis of prostaglandins and their associated compounds. Following the discovery of two isoforms of cyclooxygenase (COX1 and COX2), many studies focused on the latter, inducible isoform (COX2) as a crucial molecule in inflammatory-mediated carcinogenesis⁸⁹. Recent studies, using *Cox1*^{-/-} and *Cox2*^{-/-} mice, indicate that both of these enzymes are important in cancer development^{90–94}. Recently, a third member of the cyclooxygenase family, COX3, has been cloned and characterized⁹⁵. COX3 is selectively inhibited by pyretic drugs that alleviate pain or fever, such as acetaminophen, phenacetin, antipyrine and dipyrrone, and is potentially inhibited by some NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDs). Glycosylation is required for its activity, it is a membrane-bound 65-kDa protein (5.2 kb mRNA), and it is found mostly in the cerebral cortex and heart. The future development of *Cox3*-knockout mice will provide insight into its significance to tumorigenesis.

Human oxyradical overload diseases

We have already outlined some of the more prevalent oxyradical overload diseases above and in TABLE 1. In this section, we will focus on selected diseases as models to better understand the association between chronic inflammation and cancer in human tissues.

Haemochromatosis. The molecular changes that are associated with hepatocellular carcinoma (HCC) and other diseases that cause chronic liver injury, inflammation, hepatocellular necrosis and liver regeneration diseases, have been well characterized⁹⁶. Populations that are at high risk of developing HCC are useful models for examining the association between chronic inflammation and cancer. Included in this group are chronic hepatitis B or C carriers and patients with a genetic susceptibility to HCC, such as those with haemochromatosis. If the process of chronic liver injury and inflammation continues for many years, liver cirrhosis occurs, which then markedly increases the risk of developing HCC. These findings indicate that the modulation of cell proliferation and/or inflammatory responses that is associated with necroinflammatory diseases is responsible for the increased probability of neoplastic transformation of HCC precursor cells.

Haemochromatosis is an inherited cancer-prone disease that is characterized by excess build-up of iron in the liver, leading to tissue damage by means of oxyradical overload⁹⁷. Genes involved in hereditary haemochromatosis include *HFE*, a gene on chromosome 1q, and genes encoding the transferrin receptor 2, ferroportin and ferritin H⁹⁸. The resulting excessive accumulation of iron in hepatocytes causes hepatocellular injury, leading to fibrosis and cirrhosis (reviewed in REF. 99). The metal ions and the free radicals that are produced can induce a number of alterations in cellular macro-molecular targets, as well as point mutations in genes^{49,100–102}. Increased cell division contributes to the conversion of DNA lesions into point mutations. We have tested the hypothesis that an increase in the *TP53* mutation load in the liver of haemochromatosis patients can arise before cancer development through several different mutagenic pathways¹⁰³. Reactive aldehydes – such as 4-hydroxynonenal, malondialdehyde and crotonaldehyde – that are produced during lipid peroxidation, can generate exocyclic DNA adducts (addition of a 5–6-membered exocyclic ring on DNA bases) and point mutations^{104–106}. We have reported previously that 4-hydroxynonenal preferentially induced G:C to T:A transversions at p53 codon 249 in TK6 lymphoblastoid cells⁸⁶. There is also evidence of increased lipid peroxidation and etheno-DNA adducts (addition of a 5-membered exocyclic ring on DNA bases) in haemochromatosis patients^{107–109}.

Ulcerative colitis. UC is a chronic inflammatory disease of unknown aetiology, which is associated with an increased risk of developing colon cancer. The colonic mucosa in patients with UC shows uniform and continuous inflammation with ulceration and micro-abscess formation. Neutrophilic infiltration and inflammatory reactions have been linked with the formation of these lesions. The precursor neoplasm of UC differs in histology and morphology to that of sporadic colon cancer. Whereas adenomatous polyps are considered to be the main precursor of the sporadic disease, UC-associated cancers involve the development of epithelial dysplasia¹¹⁰. Several genetic and epigenetic changes have been described in UC that might

NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDs). This heterogeneous group of drugs primarily target the cyclooxygenase enzymes to treat pain, fever and inflammation.

BIOMARKER

A specific biochemical that provides a measure of a biological response to an endogenous or exogenous agent. The biological responses might be at the molecular, cellular or whole-organism level.

Table 2 | Key genetic polymorphisms for genes involved in DNA repair and antioxidation

	Polymorphism	Normal function	Cancer site
DNA repair genes*			
<i>OGG1</i>	S326C	Repairs 8-oxo-dG and other altered DNA bases	Oesophagus, lung, prostate
<i>XRCC1</i>	R194W	Repairs DNA-base damage and single-strand breaks	Bladder, breast, lung, SCCHN, stomach
<i>BRCA2</i>	N372H	Multiple, including DNA-damage response, DNA repair, chromatin remodelling, cell-cycle control	Breast
Antioxidant genes			
MnSOD	Val to Ala change in the -9 position	Converts O ₂ ^{••} to H ₂ O ₂	Breast
GPx	Pro198Leu	Converts H ₂ O ₂ to H ₂ O	Lung

*Information for DNA-repair genes was obtained from REF. 130. GPx, glutathione peroxidase; MnSOD, manganese superoxide dismutase; SCCHN, squamous-cell carcinoma of the head and neck.

be responsible for colon carcinogenesis¹¹¹, and these differ in frequency and timing compared with sporadic cancer^{112–115}. For instance, alterations in p53 — a late event in the molecular pathogenesis of sporadic colorectal cancer — occur earlier in the process of UC-associated carcinogenesis^{61,116}. As described above, we have identified UC patients with a high p53 mutation load and iNOS activity in areas of active inflammation⁶¹. This correlation indicates an association between high levels of free radicals and p53 mutations in the inflamed part of colon, thereby increasing the risk of developing colon cancer.

Viral hepatitis. Some 40% of all HCCs worldwide occur in hepatitis B virus (HBV)-infected individuals⁹⁶. HBV has been studied extensively, and might have several roles in HCC initiation. Many early studies focused on viral integration, because most HCCs in HBV carriers contain HBV DNA sequences apparently randomly integrated into the host chromosomal DNA¹¹⁷. This integration and

subsequent chromosomal alterations might result in the loss of tumour-suppressor genes that are necessary for cell-cycle control, differentiation and apoptosis.

Another effect of viral infection receiving increasing attention is hepatocellular necrosis, inflammation and liver regeneration. The impact of viral hepatitis, characterized by liver-cell injury or liver oedema induced by infiltration of inflammatory cells¹¹⁸, can lead to DNA damage and the modification of key proteins that are involved in carcinogenesis, which is significant in the genesis of HCC. This was shown in a transgenic mouse model of HBV, in which sustained accumulation of the DNA adduct 8-oxo-2'-deoxyguanosine (8-oxo-dG) starts early in life and increases progressively with advancing disease, leading to HCC¹¹⁹. 8-oxo-dG — one of the main DNA adducts that is formed by oxidative stress — can produce a number of missense mutations in cancer-related genes.

Many studies have focused on the influence of the hepatitis B virus x (HBx) protein on HBV-mediated hepatocarcinogenesis. HBx binds to many cellular proteins and also acts as a co-transcription factor to regulate many cellular and viral genes. The HBx and inflammatory components of hepatocarcinogenesis are probably not mutually exclusive. The HBx protein has been shown to interact with inflammation-associated molecules and activate pro-inflammatory cytokines^{120–125}. Also, both inflammatory species and HBx can deregulate cell-cycle checkpoint controls, block p53-mediated apoptosis and inhibit DNA repair, and therefore contribute to the selection of cells that are genetically unstable, some of which accumulate as unrepaired oncogenic mutants.

Although hepatitis C virus (HCV) is a significant cause of disease-associated morbidity and mortality worldwide, its role as an aetiological agent in HCC was not recognized until 1989 (REFS 126,127). Much of our knowledge about its influence on HCC has since come from large epidemiological studies. The mechanisms involved, however, are not clearly understood. Due to the impact of HCV on viral hepatitis and associated inflammation, HCV, in combination with mechanisms involved in chronic inflammation, can have potent effects on liver carcinogenesis.

Table 3 | Human chemoprevention trials in oxyradical overload diseases

Disease	Cancer inhibited/potentially inhibited	Chemopreventive agent	References
Prospective, randomized control trials			
HBV, HCV	HCC	Interferon- α	135,137–139
HBV	HCC	Sho-saiko-to (TJ-9)	161
<i>Helicobacter pylori</i>	Gastric	Ascorbic acid, β -carotene, α -tocopherol	162,163
HPV	Cervical	All- <i>trans</i> retinoic acid, β -interferon	164,165
Retrospective studies			
Crohn's disease	Colon	NSAIDs	166
UC	Colon	5-ASA, sulphalazine, mesalazine, ursudiol, folates	167–171
HBV, HCV	HCC	Neomimophagen C, glycyrrhizin, selenium, vitamins A and E, β -carotene	172–176
HPV	Cervix	Folates, carotenoids, vitamins C and E	177
<i>Helicobacter pylori</i>	Gastric	NSAIDs, aspirin	178

HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HPV, human papillomavirus; NSAIDs, non-steroidal anti-inflammatory drugs; UC, ulcerative colitis.

REVIEWS

Gene-microenvironment interactions

Molecular epidemiology of human cancer risk is the study of inter-individual variation and gene-environment interactions through a multidisciplinary effort — including epidemiology, molecular genetics, cell biology, biochemistry, statistics and bioethics. Recognizing that cancer is a genetic disease involving multiple genes, molecular epidemiology uses biomarkers of cancer risk that will elucidate multiple gene-environment interactions, in which several genes and exposures work interactively (FIG. 3).

Any inherited variation in the genetic code that alters protein expression, function or localization can increase the susceptibility to cancer. Genetic polymorphisms — changes in the nucleotide sequence that are present in at least 1% of the population — are an important example. The presence of genetic polymorphisms in genes controlling important cellular functions, such as DNA repair, apoptosis and the cell cycle, might affect cancer risk in an individual. The concentration and activity of DNA-repair enzymes are altered in inflammation^{128,129}, and cells protect themselves from the damage caused by free radicals through DNA repair. It is therefore important to assess genetic polymorphisms in DNA-repair genes in patients with chronic inflammation. Recent studies¹³⁰ have shown an association between polymorphisms in DNA-repair genes and a variety of human cancers (TABLE 2). Generation of free radicals through endogenous cellular processes creates an oxidative environment which, if not removed by the delicately balanced antioxidant enzyme

machinery, causes oxidative damage in DNA and proteins. Polymorphism of the gene encoding the antioxidant enzyme manganese superoxide dismutase (MnSOD), which converts O_2^- to H_2O_2 , alters protein trafficking and is associated with increased breast cancer risk¹³¹. Furthermore, a polymorphism at Pro198Leu in the glutathione peroxidase 1 gene — which converts H_2O_2 to water — is associated with an increased risk of lung cancer¹³². It would be interesting to extend these studies to investigate cancer-prone, oxyradical overload diseases.

Treatment and protection strategies

Targeting the genes that are involved in chronic inflammation with an increased cancer risk might provide an effective therapy. Current targets for gene therapy in acute inflammatory diseases have been reviewed elsewhere^{13,133}. They include targeting molecules that suppress inflammation (for example, tumour necrosis factor- α and - β , and interleukin-1, 4, 10 and 13), block protease activity (α_1 -antitrypsin, tissue inhibitors of metalloproteinases), reduce free radical and superoxide production (catalase, superoxide dismutase and glutathione peroxidase), inhibit apoptosis of lymphoid cell and somatic cell populations (BCL2, BCL-X_L and iNOS) or promote wound healing (such as growth factors).

Prevention of exposure or eradicating the exposure soon after it occurs is the most logical strategy for reducing the risk of cancer in oxyradical overload diseases with an environmental component. Changing behaviour (such as stopping drug addicts sharing needles and increasing the transmission of hepatitis viruses), vaccinations (for HBV and human papillomavirus) and early treatment (such as praziquantel for *Schistosomiasis* infection^{73,134} or interferon for hepatitis¹³⁵⁻¹³⁹) can also be effective. Surveillance, screening and prophylactic surgery in non-vital organs — such as the colon of UC patients — can also be useful. All approaches are complemented by chemoprevention strategies.

Chemoprevention targets pathways that are involved in the synthesis and quenching of cancer-causing molecules. In chronic inflammation, inhibiting free-radical synthesis or scavenging free radicals are attractive approaches. This can be done by dietary modulation, micronutrient supplementation and naturally or synthetically derived drugs. TABLE 3 lists some key prospective and retrospective chemoprevention trials that point towards the usefulness of this strategy to prevent cancer in patients with oxyradical overload diseases.

Conclusions and perspectives

The term 'oxyradical overload' encompasses a wide range of diseases that are associated with an increased cancer risk. Key reasons for this increased risk include DNA damage, protein modification and changes in the transcriptional activation and/or repression of genes that are responsible for cellular homeostasis. Any chronic shift in the maintenance of this cellular homeostasis can lead to permanent changes associated with carcinogenesis (FIG. 4).

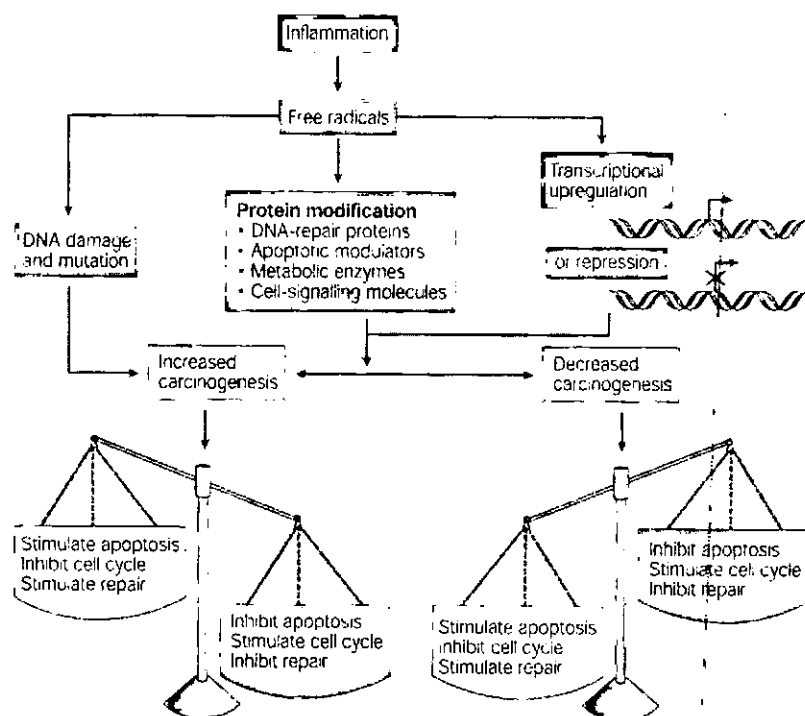


Figure 4 | Chronic inflammation and production of free radicals regulate multiple cellular processes. Free radicals released at the site of inflammation, not only damage DNA, but also modify cancer-related proteins and regulate transcription. Often, if the insult continues over a prolonged period of time, the scales are tipped in favour of increased tumorigenesis. With DNA damage and mutation, an inhibition of apoptosis, a stimulation of the cell cycle/proliferation and an inhibition of DNA repair.

Because of the complexity of free radicals, it has been difficult to delineate the specific role of each radical in carcinogenesis. We argue that NO[•] and its derivatives are not only key molecules that damage DNA, but they also modify the structure and function of proteins that are involved in the maintenance of cellular integrity and that promote angiogenesis. However, there are 'two faces' of NO[•], and further study is required before iNOS and NO[•] should be considered as targets for the prevention of chronic-inflammatory-induced carcinogenesis in humans. At present, evidence favours the use of COX2 inhibitors as chemoprevention agents⁸⁹. Combination strategies using both iNOS and COX2 inhibitors, or using single agents that target both iNOS and COX2 (for example, dexamethasone) using NO[•]-releasing NSAIDs, or targeting the NF- κ B pathway might prove to be useful approaches for reducing cancer that is associated with chronic inflammation^{140–144}. As a cautionary note, in some cases, targeting iNOS or COX2 has resulted in an exacerbation of carcinogenesis in animal models (reviewed in REFS 52,89).

Careful attention by means of chemopreventive or other regimens must be given to patients with oxyradical overload diseases to stop, or even reverse, carcinogenesis before the cancer becomes clinically observable. Towards this end, to our knowledge, there have been no retrospective or prospective chemoprevention studies published for many oxyradical overload diseases other than those listed in TABLE 3. There is a need for more long-term, prospective, randomized studies to provide insight into the agents that can protect the many people with oxyradical overload diseases from cancer development. Although the best way to test the efficacy of chemopreventive agents is by determining their ability to reduce cancer incidence, this is rarely done in a prospective manner because of the expense, time and manpower required. This is why in high cancer risk, oxyradical overload diseases, cancer as an end point is rarely used to test the efficacy of chemopreventive

agents. Instead, intermediate biomarkers can be useful as predictors of outcome. However, few markers have been properly evaluated to directly link them to cancer outcome. The impact of free radicals on DNA is thought to be the main link between free radicals and cancer formation, and as p53 is a key molecule involved in carcinogenesis, quantification of TP53 mutation load is a promising intermediate-effect marker to predict cancer risk or to test the efficacy of chemopreventive agents. Measuring the TP53 mutation load or the frequency of mutated alleles in non-tumorous tissue might indicate previous carcinogen exposure and identify individuals at increased cancer risk. As only an extremely small number of cells harbour a particular mutation at the early stage of a disease, the development of a highly sensitive genotypic assay has been important in allowing the detection of low-frequency mutations in normal-appearing human tissues, as well as in cells that are exposed to an environmental carcinogen^{145–147}. The analysis of cancer-related gene fragments in serum or other bodily specimens, such as stools, might also provide useful intermediate biomarkers^{148,149}.

Because free radicals directly modify DNA and proteins, the measurement of damage products is useful for assessing risk or for developing chemoprevention strategies in oxyradical overload diseases. These markers, as well as measuring p53 post-translational modification and accumulation²³, assess overall exposure of the body to inflammatory stress. Some promising specific markers used in the monitoring of nitrosative stress and chemoprevention of this stress include *N*-nitrosoproline, *N*-nitrosamino acid and NO₃⁻ in urine or plasma; 8-oxo-dG, 8-nitro-soguanosine, exocyclic etheno- and malondialdehyde-DNA adducts in leukocytes or target tissue; and 3-nitrotyrosine protein adducts^{12,52,150,151}. So far, the consequences of these effect markers on human carcinogenesis are unknown and need to be evaluated so that useful chemopreventive strategies can be properly assessed.

- 1 Coussens, L. M. & Werb, Z. Inflammation and cancer. *Nature* **420**, 860–867 (2002).
- 2 Nathan, C. Points of control in inflammation. *Nature* **420**, 846–852 (2002).
- 3 Christen, S., Hagen, T. M., Shigenaga, M. K. & Ames, B. N. *Microbes and Malignancy: Infection as a Cause of Cancer* 35–88 (Oxford Univ. Press, New York, 1999).
- 4 Ames, B. N., Gold, L. S. & Wiet, W. C. The causes and prevention of cancer. *Proc. Natl Acad. Sci. USA* **92**, 5258–5265 (1995).
- 5 Cerutti, P. A. & Trumb, B. F. Inflammation and oxidative stress in carcinogenesis. *Cancer Cells* **3**, 1–7 (1997).
- 6 Shelton, D., Smith, S. J. & Brown, P. O. A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization. *Genome Res.* **6**, 639–645 (1996).
- 7 Cerutti, P. A. Prooxidant states and tumor promotion. *Science* **227**, 375–381 (1985).
- 8 Phillips, L. L. Effect of free radicals on chromosomes of barley. *Science* **124**, 889–890 (1956).
- 9 Key study showing that free radicals damage DNA. Wiseman, H. & Halliwell, B. Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *Biochem. J.* **313**, 17–29 (1996).
- 10 Marshall, H. F., Merchant, K. & Stanler, J. S. Nitrosation and oxidation in the regulation of gene expression. *FASEB J.* **14**, 1899–1900 (2000).
- 11 Aulak, K. S. *et al.* Proteomic method identifies proteins nitrated *in vivo* during inflammatory challenge. *Proc. Natl Acad. Sci. USA* **98**, 2056–2061 (2001).
- 12 Important report of vast protein nitration following an inflammatory challenge in animals. Marnett, L. J. Oxyradicals and DNA damage. *Carcinogenesis* **21**, 361–373 (2000).
- 13 Whiteman, M., Hooper, D. C., Scott, G. S., Kopravsky, H. & Halliwell, B. Inhibition of hypochlorous acid-induced cellular toxicity by nitric oxide. *Proc. Natl Acad. Sci. USA* **99**, 12067–12068 (2002).
- 14 Gustledge, J. M. & Halliwell, B. Free radicals and antioxidants in the year 2000: A historical look to the future. *Ann. NY Acad. Sci.* **899**, 136–147 (2000).
- 15 Beckman, J. S. & Koppen, W. H. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am. J. Physiol.* **271**, C1424–C1437 (1996).
- 16 Espey, M. G. *et al.* A chemical perspective on the interplay between NO[•], reactive oxygen species, and reactive nitrogen oxide species. *Ann. NY Acad. Sci.* **962**, 195–205 (2002).
- 17 Pryor, W. A. Oxy-radicals and related species: their formation, fates, and reactions. *Annu. Rev. Physiol.* **48**, 657–667 (1986).
- 18 Radi, R. Peroxynitrite: reactions and diffusion in biology. *Chem. Res. Toxicol.* **11**, 720–721 (1998).
- 19 Thomas, D. D., Lu, X., Kantrow, S. P. & Lancaster, J. R. Jr. The biological lifetime of nitric oxide: implications for the perovascular dynamics of NO and O₂. *Proc. Natl Acad. Sci. USA* **98**, 355–360 (2001).
- 20 Stadtman, E. R. & Levine, R. L. Protein oxidation. *Ann. NY Acad. Sci.* **899**, 191–208 (2000).
- 21 Lüscher, J. P., Pate, R. P. & O'Donnell, V. B. Pathophysiology of nitric oxide and related species: free radical reactions and modification of biomolecules. *Mol. Aspects Med.* **19**, 221–357 (1998).
- 22 Squadrito, G. L. & Pryor, W. A. Oxidative chemistry of nitric oxide: the roles of superoxide, peroxynitrite, and carbon dioxide. *Free Radic. Biol. Med.* **25**, 342–403 (1993).
- 23 Holseth, J. J. *et al.* Nitric oxide-induced cellular stress and p53 activation in chronic inflammation. *Proc. Natl Acad. Sci. USA* **100**, 143–148 (2003).
- 24 Nakaya, N., Lowe, S. W., Taya, Y., Chenchik, A. & Lyskovsky, G. Specific pattern of p53 phosphorylation during nitric oxide-induced cell cycle arrest. *Oncogene* **19**, 6369–6375 (2000).
- 25 Xu, W., Liu, L., Smith, G. C. & Charles, I. Nitric oxide upregulates expression of DNA-PKcs to protect cells from DNA-damaging anti-tumor agents. *Nature Cell Biol.* **2**, 339–345 (2000).
- 26 Key study showing that nitric oxide activates kinases. Wink, D. A. *et al.* Chemical biology of nitric oxide: regulation and protective and toxic mechanisms. *Curr. Top. Cell Regul.* **34**, 159–187 (1996).
- 27 Pervin, S., Singh, R. & Chaudhuri, G. Nitric oxide-induced cytostasis and cell cycle arrest of a human breast cancer cell line (MDA-MB-231): potential role of cyclin D1. *Proc. Natl Acad. Sci. USA* **98**, 3583–3588 (2001).
- 28 Melino, G. *et al.* S-nitrosylation regulates apoptosis. *Nature* **388**, 432–433 (1997).

This report showed that nitric oxide changes the structure and function of key apoptosis proteins.

REVIEWS

29. Jaiswal M, Li Russo N, F. Nishikawa N, A. Nishikawa Y, A. Nishikawa G, J. Hunter O. A protein involved in the repair of 8-oxoguanine is inhibited by nitric oxide. *Cancer Res* 61: 6389-6393 (2001).
30. Wink D A & Leavelle J. The Eps protein, a DNA repair enzyme, is inhibited by the peroxyl radical nitric oxide in vitro and in vivo. *Carcinogenesis* 15: 2125-2129 (1994).
31. Jenkins D C et al. Roles of nitric oxide in tumor growth. *Proc Natl Acad Sci USA* 92: 4392-4396 (1995).
Key study showing the dual roles of nitric oxide in animals.
32. Manda H, Noguchi Y, Sato K & Asake T. Enhanced vascular permeability in tumor is mediated by nitric oxide and inhibited by both hemodynamic and metabolic synthesis inhibitor. *Jpn J Cancer Res* 85: 327-334 (1994).
33. Tizer G M, Pese V E & Singh D J. Inhibition of nitric oxide synthase induces a selective reduction of tumor blood flow that is reversible with L-arginine. *Cancer Res* 57: 948-953 (1997).
34. Timpl S, Burney S & Tannenbaum S R. DNA damage by nitric oxide. *Chem Res Toxicol* 9: 821-827 (1996).
35. Oshima H & Bartsch H. Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. *Mutat Res* 305: 253-264 (1994).
36. Wink D A et al. The effect of various nitric oxide-donor agents on hydrogen peroxide-mediated toxicity: a direct correlation between nitric oxide formation and protection. *Arch Biochem Biophys* 331: 241-248 (1996).
37. Hecker R, Poncek T, Grabner R & U. Nitric oxide inhibits proliferation of human endothelial cells via a mechanism independent of cGMP. *Atherosclerosis* 144: 49-52 (1999).
38. Karaman Y, Takada T, Sakai R & Mizuno K. Effect of nitric oxide on human endothelial cells. *Mol Cell Biochem* 194: 1-10 (1999).
39. Jaiswal M, Li Russo N, F. Nishikawa N, A. Nishikawa Y, A. Nishikawa G, J. Hunter O. A protein involved in the repair of 8-oxoguanine is inhibited by nitric oxide. *Cancer Res* 61: 6389-6393 (2001).
40. Wink D A et al. The effect of various nitric oxide-donor agents on hydrogen peroxide-mediated toxicity: a direct correlation between nitric oxide formation and protection. *Arch Biochem Biophys* 331: 241-248 (1996).
41. Hecker R, Poncek T, Grabner R & U. Nitric oxide inhibits proliferation of human endothelial cells via a mechanism independent of cGMP. *Atherosclerosis* 144: 49-52 (1999).
42. Dimmeler S, Haendeler J, Nicks M & Zeher A M. Suppression of apoptosis by nitric oxide via inhibition of interleukin-1 β -converting enzyme (ICE)-like and cysteine protease (caspase-3) activities. *J Exp Med* 185: 601-607 (1997).
43. Lin J, Bornbeck C A, Yang S, Kim Y M & Blier T R. Nitric oxide suppresses apoptosis via inhibiting caspase activation and mitochondrial dysfunction in cultured neutrophils. *J Biol Chem* 274: 17325-17333 (1999).
44. Kim Y M, Chung H T, S. Chung R L & Blier T R. Cellular membrane content is a determinant of nitric oxide-mediated apoptosis: nitric oxide and caspase inhibition. *J Biol Chem* 275: 10244-10249 (2000).
45. Chung H T, Pae H O, Choi B M, Blier T R & Kim Y M. Nitric oxide as a regulator of apoptosis. *Biochem Biophys Res Commun* 282: 1075-1079 (2001).
46. Mello G et al. A hypoxia-responsive element mediates a novel pathway of activation of the inducible nitric oxide synthase promoter. *J Exp Med* 182: 1683-1693 (1995).
47. Chen B & Deen W M. Analysis of the effects of cell spacing and fluid depth on nitric oxide and its oxidant products in cell cultures. *Chem Res Toxicol* 14: 135-147 (2001).
48. Burney S, Niles J C, Dedon P C & Tannenbaum S R. DNA damage in deoxyribose and oligonucleotides mediated by peroxynitrite. *Chem Res Toxicol* 12: 613-620 (1999).
49. Burney S, Lauterbach J L, Niles J C, Witek J S & Tannenbaum S R. The chemistry of DNA damage from nitric oxide and peroxynitrite. *Mutat Res* 424: 37-44 (1999).
50. Grollman A P, Auer R F, F. Nishikawa N, A. Nishikawa Y, A. Nishikawa G, J. Hunter O. A protein involved in the repair of 8-oxoguanine is inhibited by nitric oxide. *Cancer Res* 61: 6389-6393 (2001).
51. Schmidt K, Dörner W, K. P. A. Nishikawa Y, A. Nishikawa G, J. Hunter O. A protein involved in the repair of 8-oxoguanine is inhibited by nitric oxide. *Cancer Res* 61: 6389-6393 (2001).
52. Hines J, J. Hussain S P, Wogan G N & Hines J C. Nitric oxide in cancer and its prevention. *Free Radic Biol Med* (in press).
53. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
54. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
55. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
56. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
57. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
58. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
59. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
60. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
61. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
62. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
63. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
64. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
65. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
66. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
67. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
68. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
69. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
70. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
71. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
72. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
73. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
74. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
75. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
76. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
77. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
78. Zhuang J C, Wright T L, de Rojas-Walker T, Tannenbaum S R & Wogan G N. Nitric oxide-induced mutations in the *hprt* gene of human lymphoblastoid TK6 cells and in *Salmonella typhimurium*. *Environ Mol Mutagen* 35: 41-47 (2000).
79. Kumar A & Deery W D. The therapeutic potential of nitric oxide in infection and inflammation. *Ann Rev Med Ther Exp* 48: 313-338 (2000).
80. Kumar A, Lerner J, Krenkel D, Reznayk K, A. Nishikawa Y, A. Nishikawa G, J. Hunter O. A protein involved in the repair of 8-oxoguanine is inhibited by nitric oxide. *Cancer Res* 61: 6389-6393 (2001).
81. Berg D et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).
82. Kullberg M C et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).
83. Kullberg M C et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).
84. Kullberg M C et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).
85. Kullberg M C et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).
86. Kullberg M C et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).
87. Kullberg M C et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).
88. Kullberg M C et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).
89. Kullberg M C et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).
90. Kullberg M C et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).
91. Kullberg M C et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).
92. Kullberg M C et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).
93. Kullberg M C et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).
94. Kullberg M C et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).
95. Kullberg M C et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).
96. Kullberg M C et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).
97. Kullberg M C et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).
98. Kullberg M C et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).
99. Kullberg M C et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).
100. Kullberg M C et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).
101. Kullberg M C et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).
102. Kullberg M C et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).
103. Kullberg M C et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).
104. Kullberg M C et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).
105. Kullberg M C et al. Enteric dysfunction in mice with intestinal inflammation: a possible role for nitric oxide. *Gastroenterology* 108: 1010-1020 (1995).