



EUROTOX Educational Course hosted by
Croatian Society of Toxicology:

Principles of Toxicology

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Carcinogenesis: Chemical Carcinogenesis

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Cancer: malignant neoplasm, disease characterized by mutation, modified gene expression, cell proliferation, and aberrant cell growth;
Second leading cause of death.

Females: Breast cancer

Males: lung (bronchial) cancer

Causes: infectious agents, radiation, and chemicals.

70% to 90% of all human cancers linked to environmental, dietary, and behavioral factors.



Historical Overview

- 1775 - Pott** - increased occurrence of scrotal and nasal cancer among chimney sweeps
- concluded: chimney soot was the causative agent
- 1890 - Von Hansemann** - all cancer cells bear aberrant chromosomes
- 1892 - Butlin** - duration of exposure influences risk of developing cancer
- 1914 - Boveri** - cell possesses cell division controlling „elements” as parts of the chromosomes; concluded: chromosomes play role in cancer formation
- 1915 - Kennaway, Hieger** - dibenz(a,h)anthracene in coal tar is skin cancer inducing agent
- 1928 - Meisel** – chemicals may be mutagens
- 1960 - Nowell, Hungerford** - each leukemia cell bears aberrant chromosomes; detection of Philadelphia chromosome
- 1951-1983 Miller et al.** - established the role of chemical structure and metabolism in the “activation” of carcinogens
- 1988 - Heim et al.** - proved: accumulation of chromosomal damage needed for cancer formation

Terms in Carcinogenesis

Carcinogenesis: new or autonomous growth of tissue (neoplasia) resulting in formation of a lesion named neoplasm:

benign: expansive slow growth do not invade surrounding tissues

malignant: invasive growth, invading other organs via metastasis.

Cancer: malignant neoplasm, disease characterized by mutation, modified gene expression, cell proliferation, and aberrant cell growth;

Second leading cause of death.

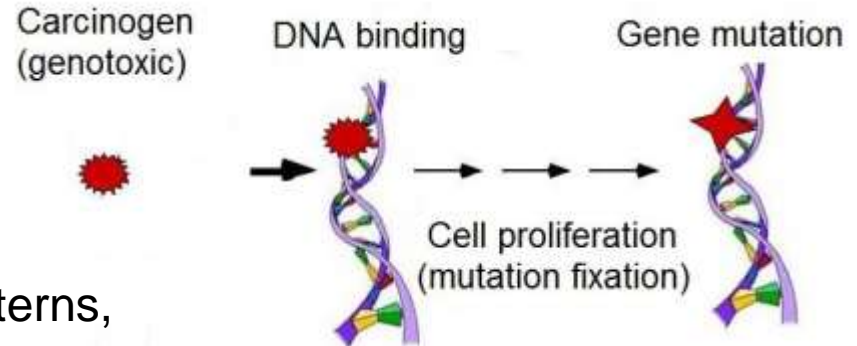
Carcinogens: physical or chemical agent that causes or induces neoplasia

Tumor: lesion characterized by swelling or increase in size, may or may not be neoplastic

Types of carcinogens with regards to the mechanism of induction:

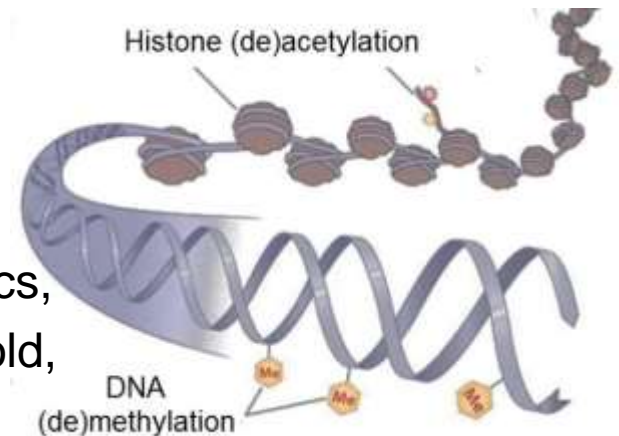
- **genotoxic: interact with DNA resulting in mutation:**

- frequently mutagenic in a dose-responsive manner,
- exhibiting low-dose linear patterns, approximating a straight line at very low doses without a threshold,
- lack of reversibility of effect upon removal of the agent.



- **nongenotoxic (epigenetic): modify gene expression without damaging DNA:**

- create a situation in a cell making it more susceptible to DNA damage from xenobiotics,
- exhibit a nonlinear dose response - threshold,
- reversible dose response pattern upon removal of the agent
- species, strain, tissue specificity



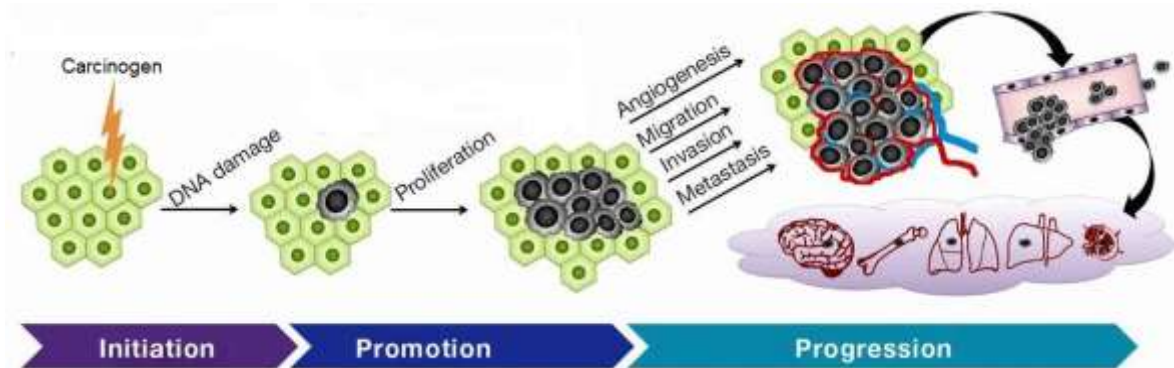
Carcinogens classification with regards to their mode of action:

- **primary (direct acting) carcinogens** - no need for metabolic activation to act as cancer causative agent
- **secondary carcinogens (indirect acting)** - have to be metabolically activated
- **co-carcinogens** - increase carcinogenic potential by stimulating biotransformation of a certain substance
- **promoters** - suppress apoptosis; stimulate proliferation

The process of carcinogenesis is a multistage and multistep process
involvs modification and mutation to a number of cellular processes

Stages in multistage carcinogenesis:

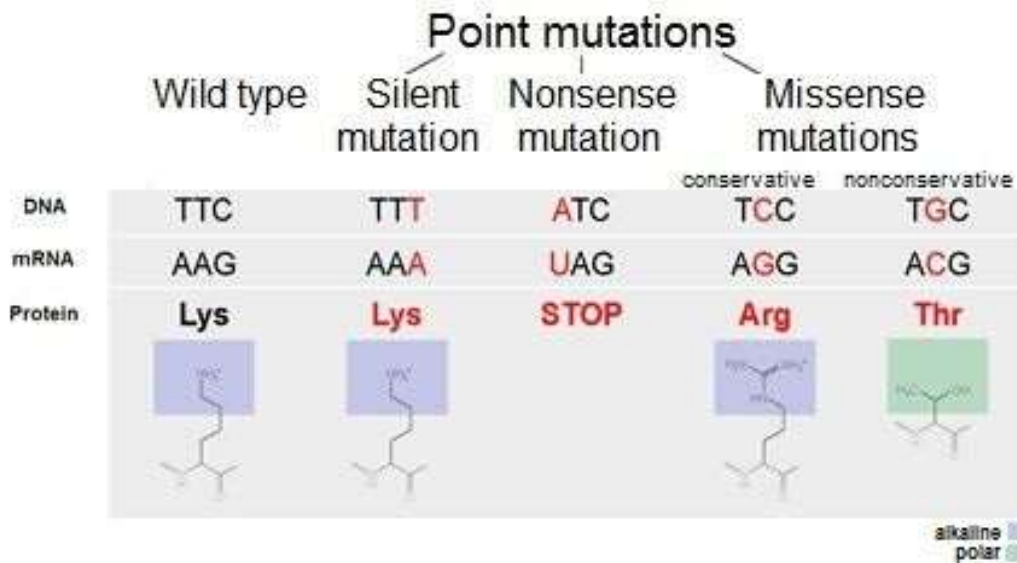
- initiation
- promotion
- progression



Genotoxic Carcinogenesis

Initiation Stage

- first stage of cancer process
 - rapid
 - Irreversible
- first step: DNA modification by initiator (**binding of carcinogen or its functional group**)
- middle step: single **DNA replication** without previous or simultaneous DNA repair
- final step: formation of stable heritable changes in the cell DNA = **mutations**



Frameshift mutations

1. Base insertions



2. Base deletions



Initiation by itself not sufficient for neoplasm formation

- potential outcomes for initiated cell:
 - remain in nondividing state through influences by growth control either via normal surrounding cells or through endocrine influence
 - mutations are incompatible with viability - initiated cell deleted through apoptotic mechanisms
 - through stimuli such as intrinsic factors or from chemical exposure, initiated cell undergo division resulting in the growth in the proliferation
- besides initiation via carcinogen binding, induction of continual stress, resulting in continual cell proliferation, can also produce new mutated, initiated cells:
- indirectly, sustained cell proliferation results in the formation of mutations;
 - usually, continuous stress is ascribed to the neoplasm promotion stage as an epigenic carcinogenesis pathway

Initiators Modes of Action

1978 - Millers concluded: **electrophilic molecules** predicted to be **initiators** – affinity to covalently bind to **nucleophilic centers in DNA**

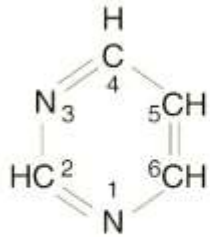
1951-1983 Miller et al. - there is a strong **correlation and regularity** between **chemical structure** of the molecule and its

- biological activity (potentially direct **carcinogenicity**),
- pathway of metabolic transformation (potentially **activation** and indirect **carcinogenicity**)

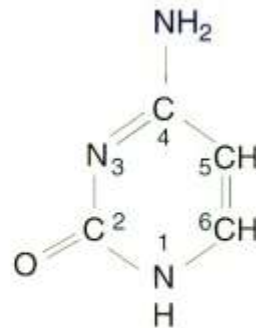
There research = basis for establishing rules in prediction of possible carcinogenicity of substances - **SAR (Structure-Activity Relationship)**.

SAR assigns electrophilic centers in molecules as „**alerts**”

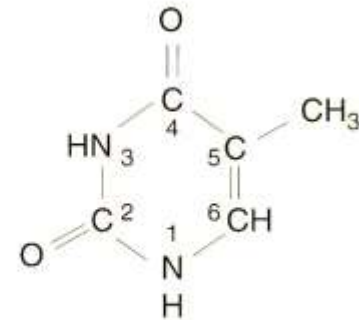
Recapitulation: Nitrogenous Bases in DNA:



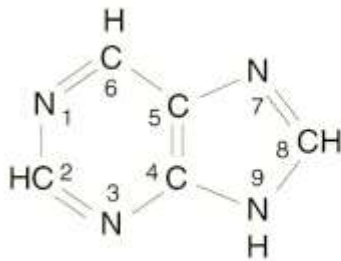
Pyrimidine



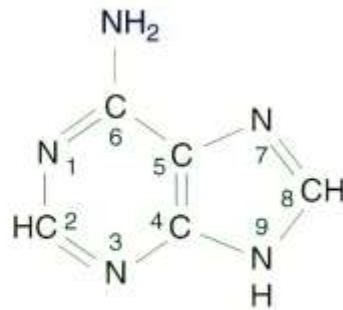
Cytosine (C)



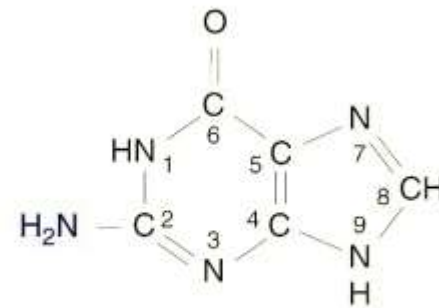
Thymine (T)



Purine



Adenine (A)



Guanine (G)

Electrophilic Centers in Direct (primary) Initiators

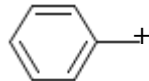
- do not require metabolic activation to become able to covalently bind to DNA bases

Alkyl ions $R-CH_2^+$

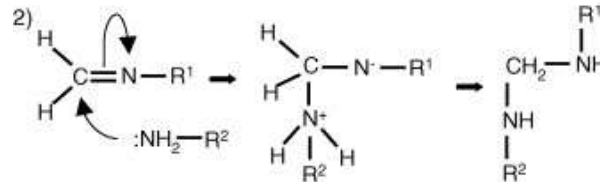
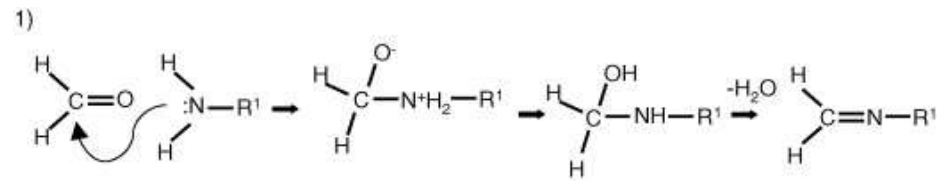
Aryl ions



Benzyl ions



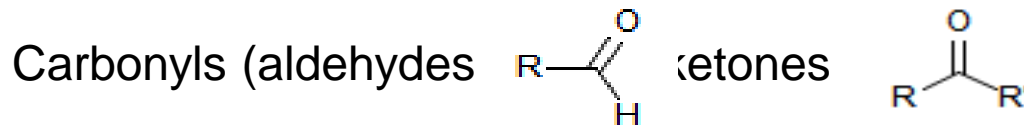
Formaldehyde ion $+CH_2-O^-$



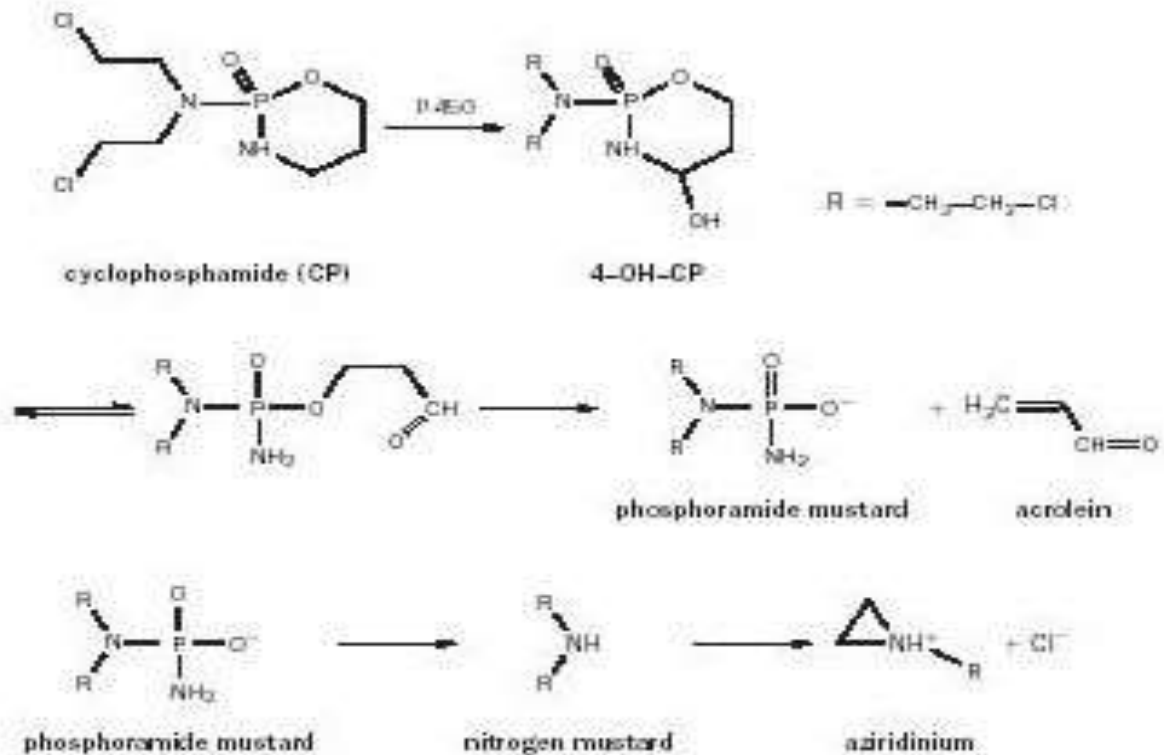
Electrophilic Centers in Indirect (secondary) Carcinogens

- **do require** metabolic activation to become able to covalently bind to DNA bases

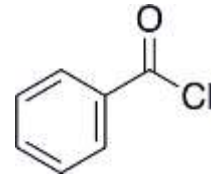
α , β -unsaturated carbonyls / cyrboxylates



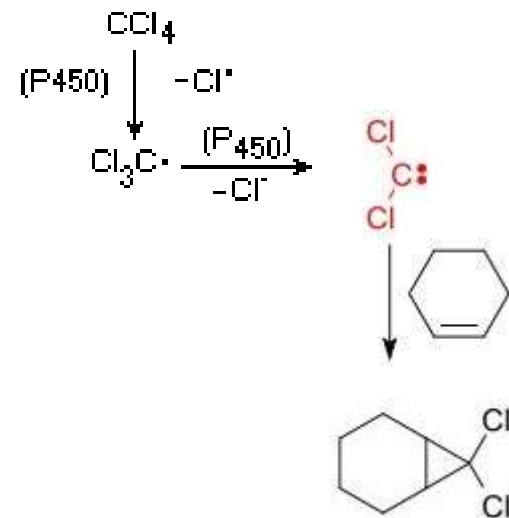
Carboxylates



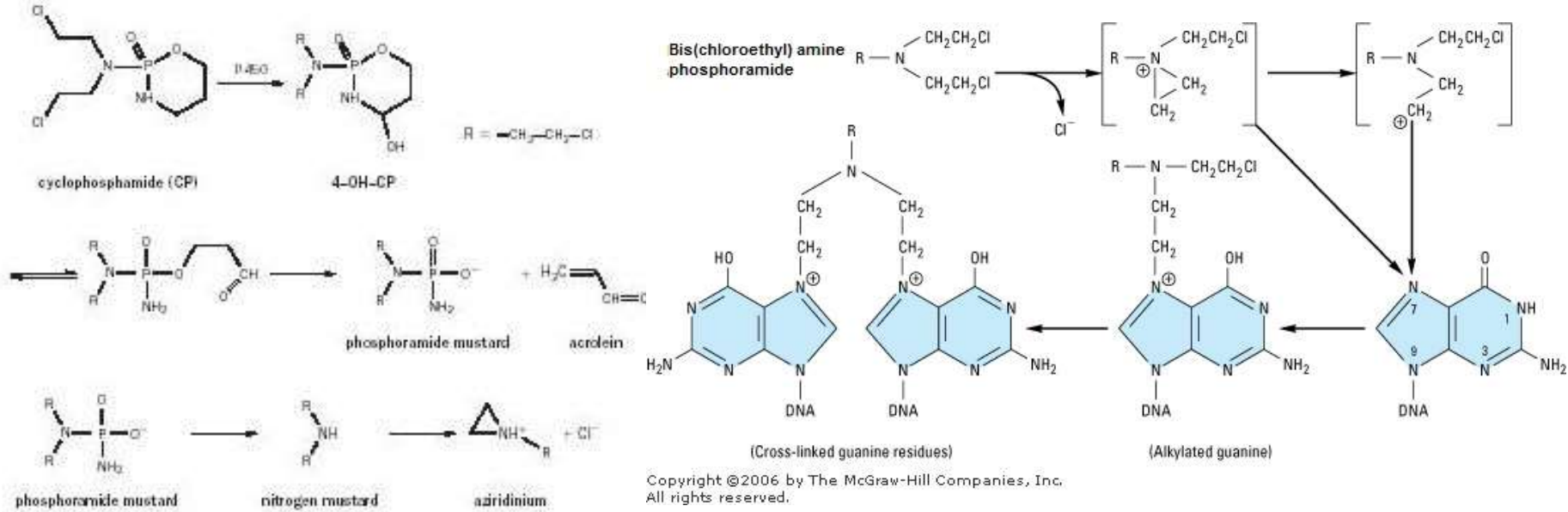
Acyl halides (Benzoyl chloride)
(activation by CYP450)



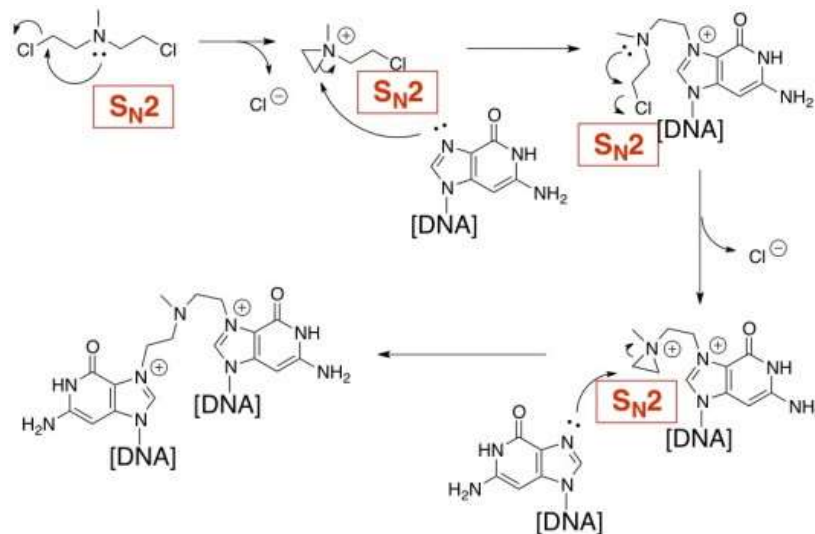
Carbenes (Carbon tetrachloride)



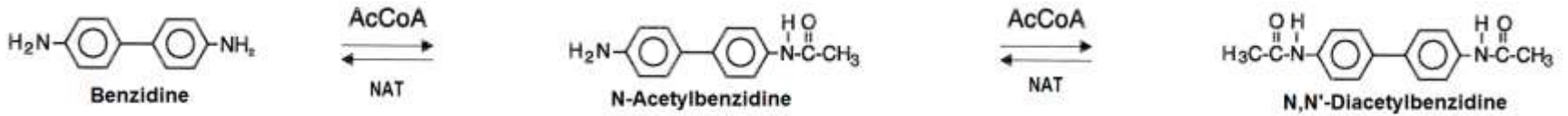
Aziridinium ion (Cyclophosphamide)



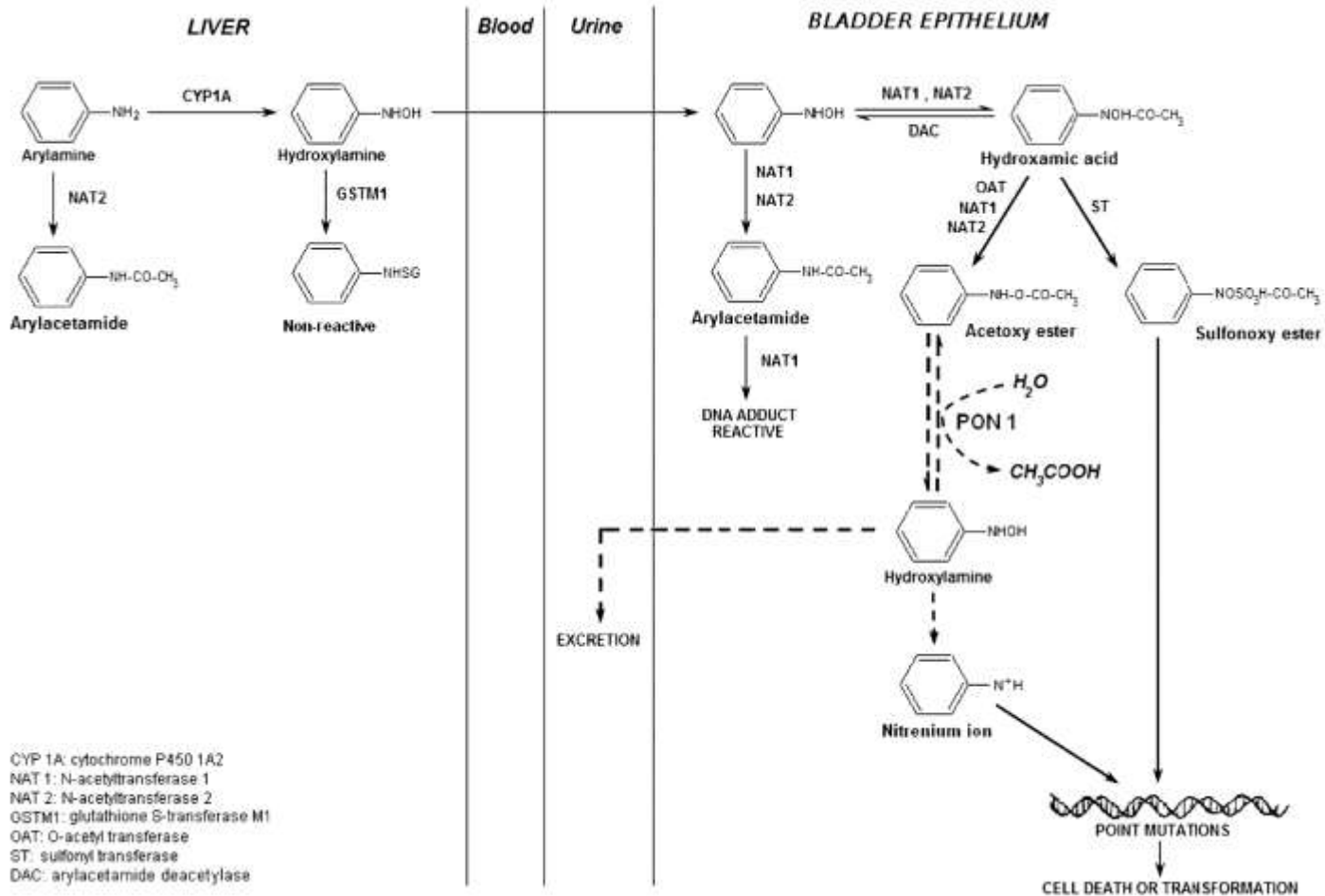
(Nitrogen yperit (mustard) - bis(2-chloroethyl)methylamine)



Nitrogen radical (Benzidine)

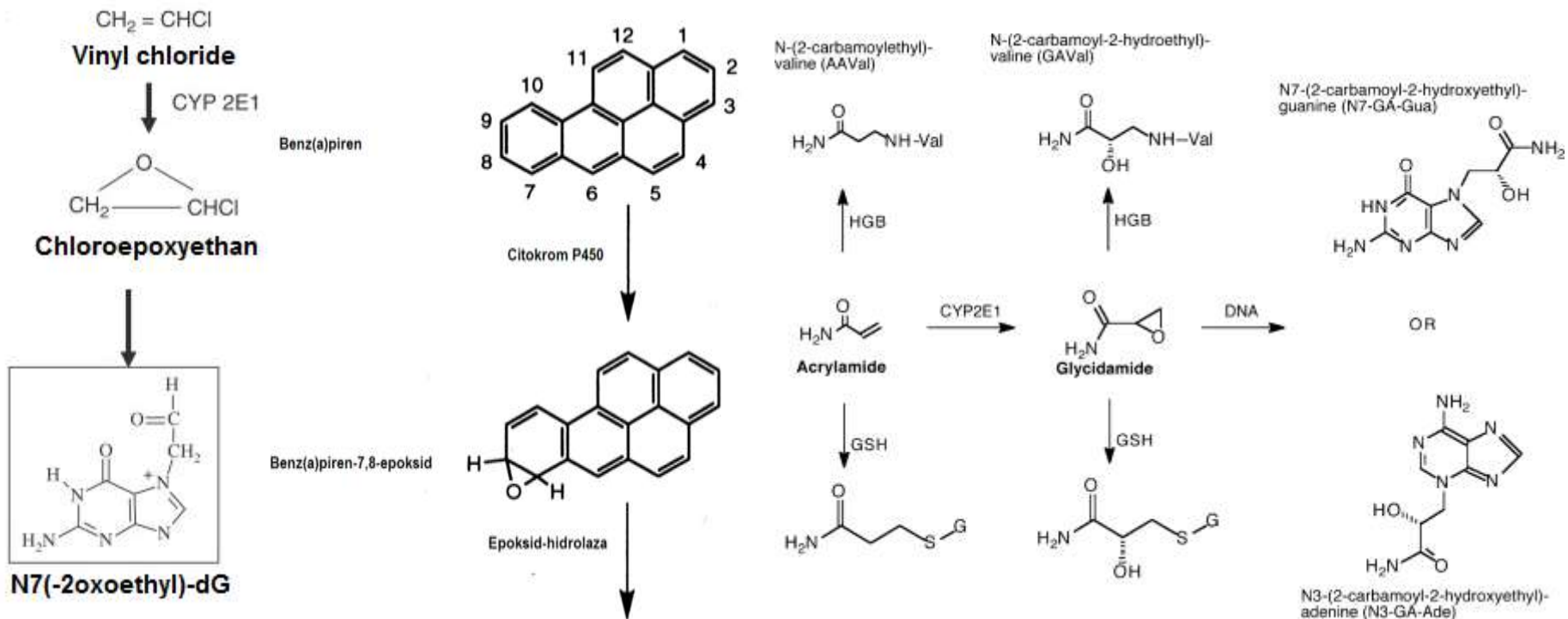
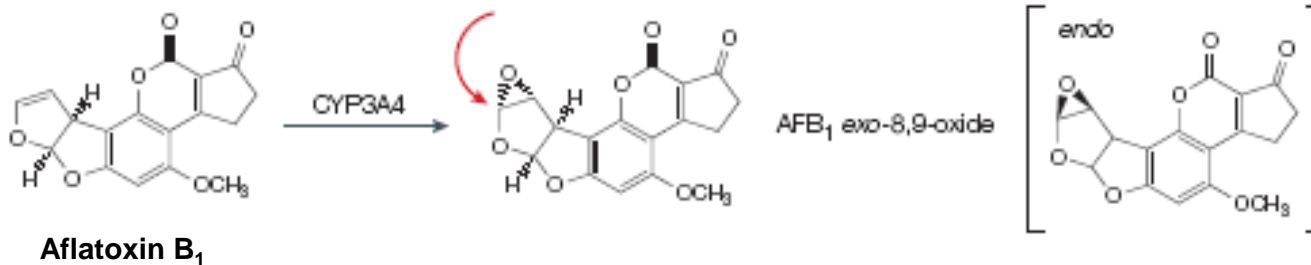


(Phenylamine)



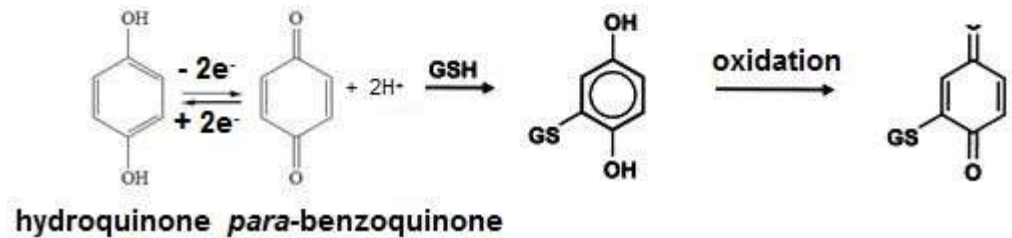
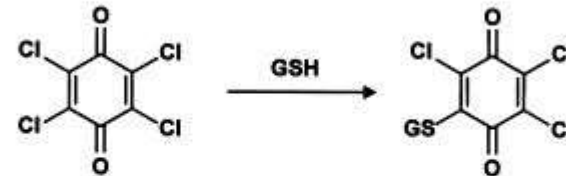
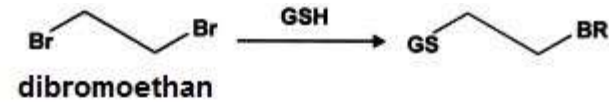
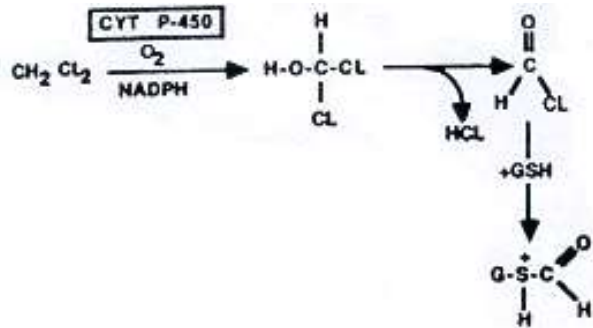
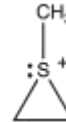
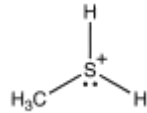
Peroxy radicals $R-O-O \cdot$

Epoxides (Aflatoxin, vinyl chloride, benzo(a)pyrene, acrylamide)



Sulfonium ion

(Dichlormethane, 1,2-dibromoethane, tetrachloro-1,4-benzoquinone)



Characteristics that Mediate Electrophilic Center Activity

Still, **though electrophilic center** is present in the molecular structure, the molecule **may not be carcinogenesis initiator**.

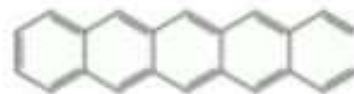
Surrounding functional groups as well as **overall characteristics** of the molecule **influence** stability and reactivity of electrophilic centers in the structure:

1. **Molecular mass beyond 1000** – low probability of DNA binding or interaction with metabolism enzymes – low level of absorption into organism. Should **not** be either highly **hydrophilic or lipophilic**.
2. Arrangement of aromatic rings - PAH, TCDD (dioxin), aflatoxin B₁ – **planar in structure with less than 4 aromatic rings in a line**, surface below 100 Å = molecule initiator

PAHs of symmetrical structure and linearly bound rings \neq initiators

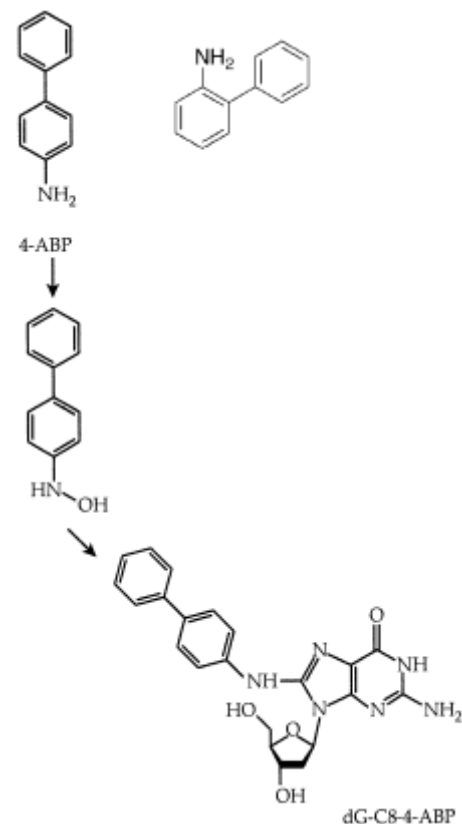
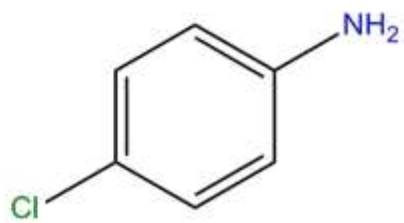
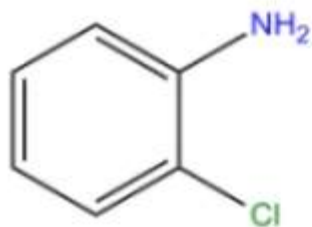


benzo(a)pyrene



pentacene

3. Only molecules planar in structure may undergo **N-hydroxylation** (1. step of metabolic activation) – increases carcinogenic potential:
- example: 4-aminobiphenyl
 - opposite: 2-aminobiphenyl loss of planarity – amino group may **not** be **accessed** by **N-hydroxylase** ≠ **carcinogen**



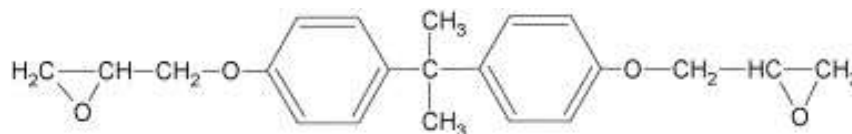
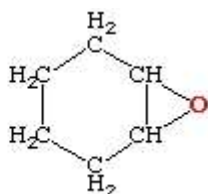
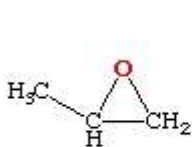
4. **Substitution** – molecules with chlorine, methyl i methoxy groups in **ortho** substitution with regards to amino group are of increased ability to form **DNA adducts**.

However, **size of the substituent** is reversely reciprocal with adduct formation – sterically hinder N-hydroxylation or N-acetylation of neighboring amino group

5. Flexibility in structure, number and distance between electrophilic centers:

Epoxides on aliphatic rings which are more rigid than aliphatic chains are **less reactive** (chain structure is more flexible which facilitates interaction with DNA).

Diepoxides are **more reactive** than mono ones. As **higher distance** between epoxy groups as higher reactivity = ability of **cross-linking DNA** bases within or between the chains.

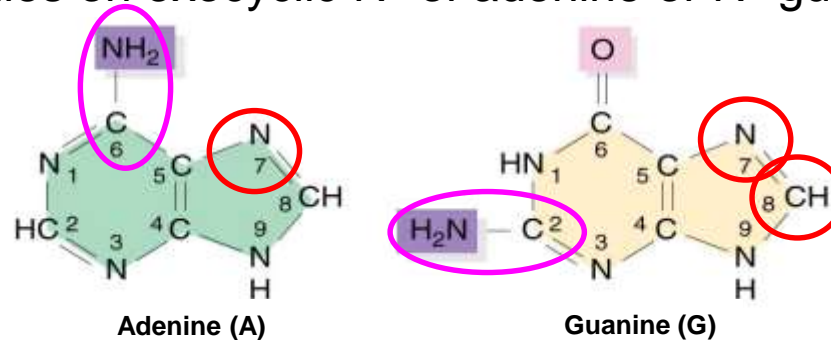


5. **Resonance stabilization** of electrophilic center.

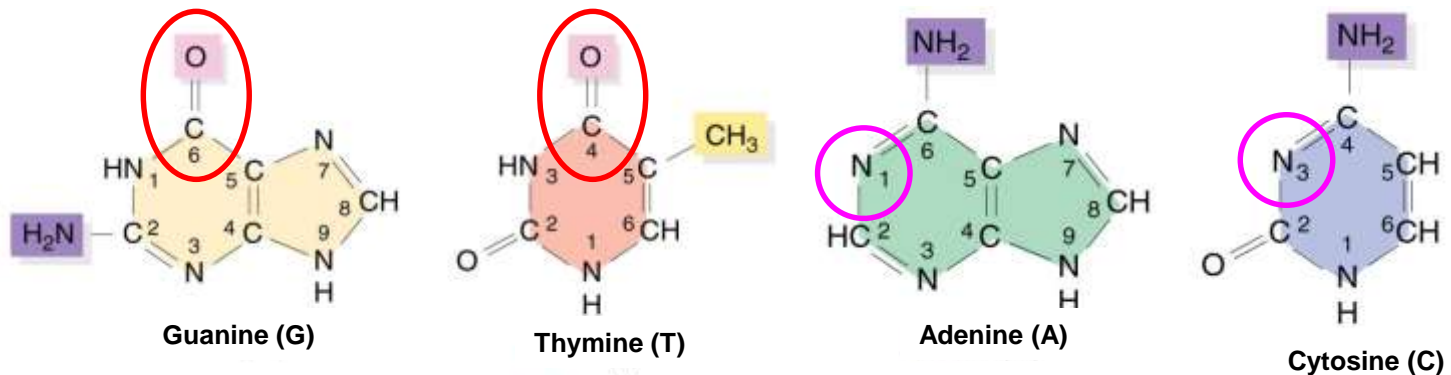
Due to high reactivity electrophilic center would be neutralized by glutathione. Neighboring double bond or aryl group **stabilize electrophilic centers** by resonance which enhances the ability to reach and interact with DNA.

Most Frequently Attacked Nucleophilic Centers in DNA

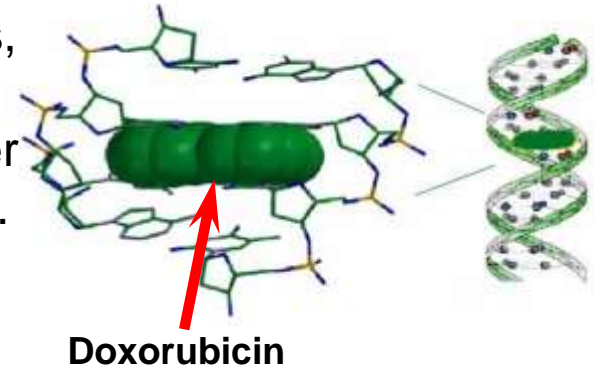
Adduct formation - **N7** of adenine and guanine, **C8** of guanine; polycyclic aralkylated molecules on exocyclic **N⁶** of adenine or **N²** guanine → substitution



Alkylations – exocyclic **O⁶** of guanine and **O⁴** thymine
N1 adenine or N3 cytosine



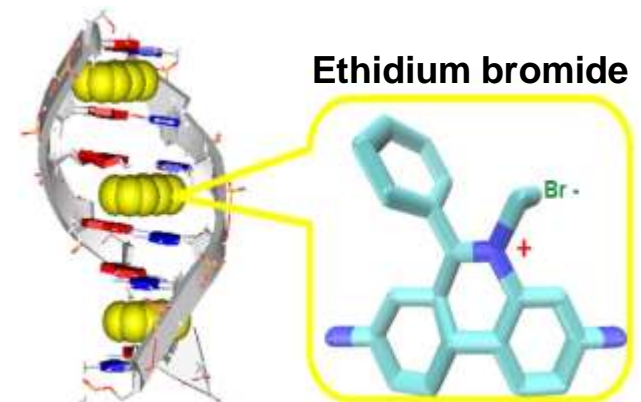
Beside molecules that **covalently** bind to DNA bases, mutations may be induced, and carcinogenesis initiated, by agents that interact with DNA by Van der Waals forces **intercalating** between two base-pairs.



Example: doxorubicin, ethidium bromide

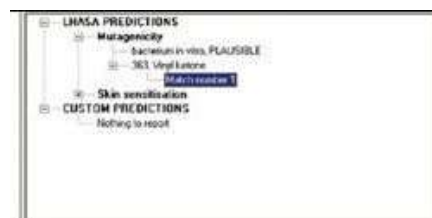
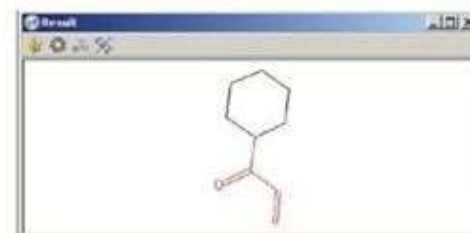
Condition: planar molecule

Mode of action: topoisomerase II poison by DNA replication → base insertion = frameshift mutations



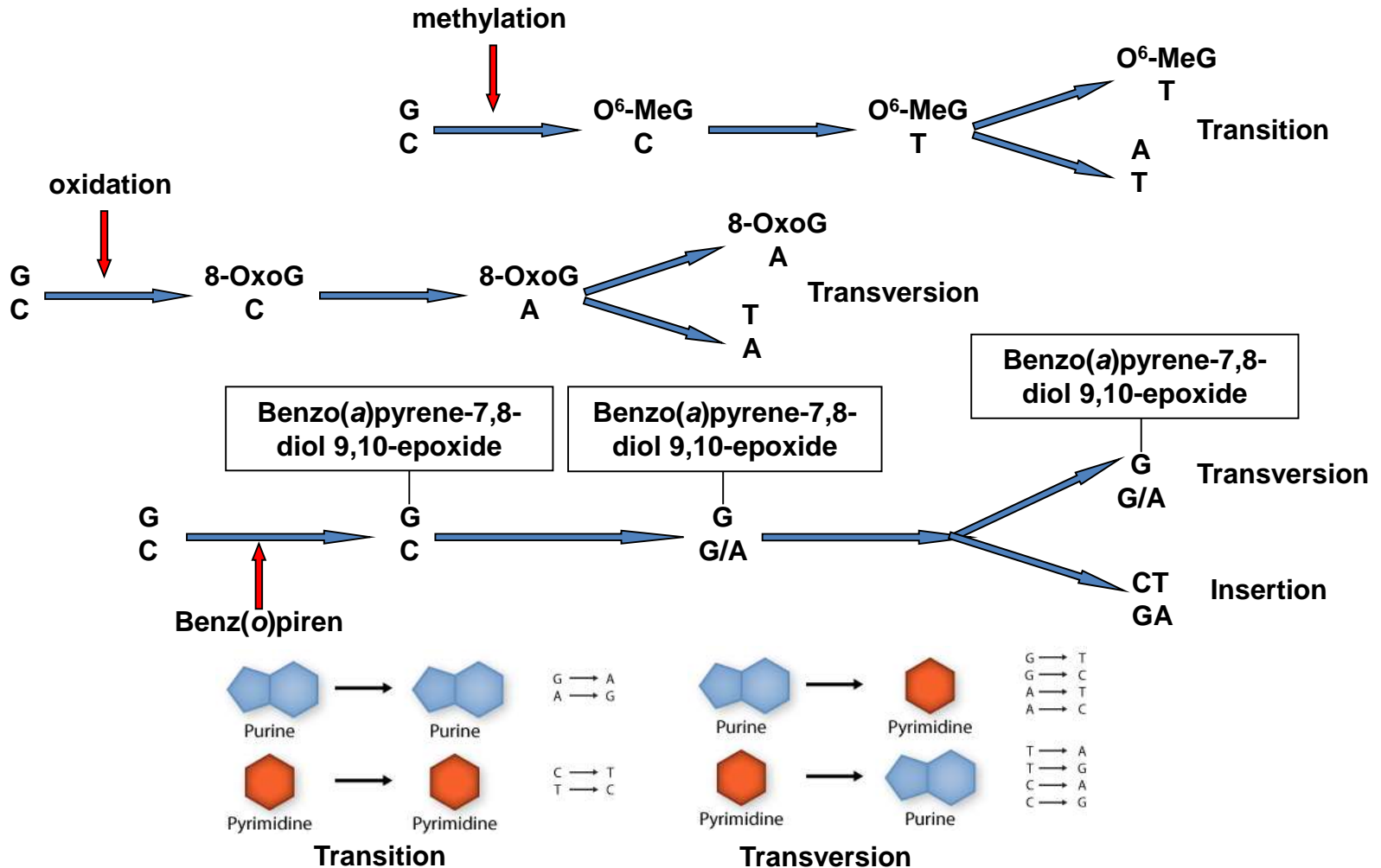
Quantitative Structure-Activity Relationship (QSAR) Analysis

- **first line** in assessment and **detection** of possible carcinogenesis **initiators**
- **software analyses:**
 - molecular structure of the molecule,
 - relations between functional groups and
 - their mutual influence and influence of entire chemical structure of the molecule,
 - possible pathways and products of their metabolic activation
- **searches database** with known carcinogens and **compare results** of the analysis of molecule of the concern
- **indicates „alerts” and „biophores”**
- Estimates their carcinogenic potential by using **multivariate statistical methods quantitatively comparing** detected characteristics of „alerts” with biological activity of known carcinogens



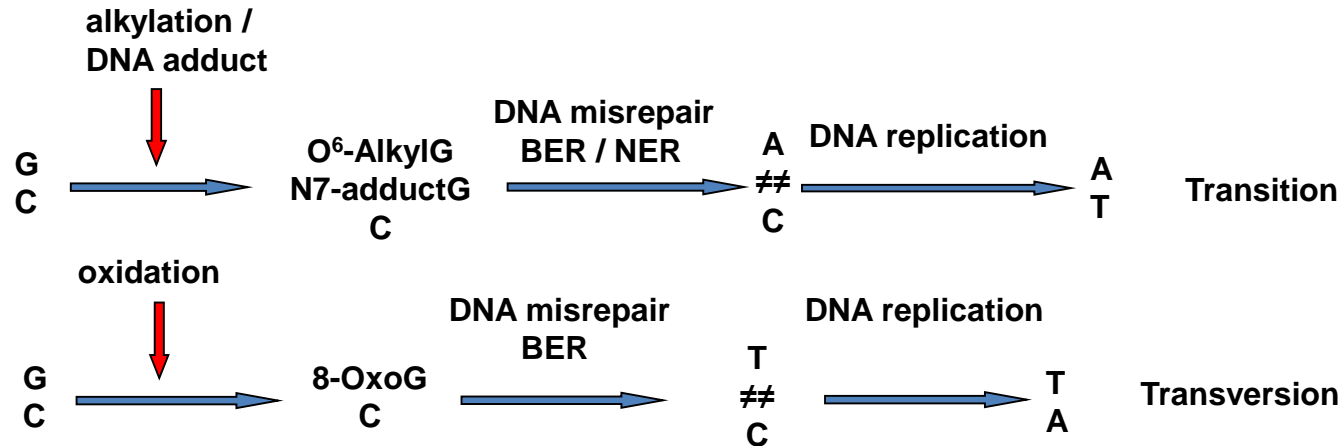
Fixation of Primary DNA Damage to Mutation

1. DNA replication:



Fixation of Primary DNA Damage to Mutation

2. DNA misrepair:



Point mutations

| | Wild type | Silent mutation | Nonsense mutation | Missense mutations | |
|---------|-----------|-----------------|-------------------|--------------------|-----------------|
| | | | | conservative | nonconservative |
| DNA | TTC | TTT | ATC | TCC | TGC |
| mRNA | AAG | AAA | UAG | AGG | ACG |
| Protein | Lys | Lys | STOP | Arg | Thr |

alkaline polar

Frameshift mutations

1. Base insertions

3'-AAA GCT ACC ATA TCG GTT A-5'
5'-TTT CGA TGG TAT AGC CAA T-3'
N-Phe Arg Trp Tyr Ser Gln

2. Base deletions

GCTA
CGAT

3'-AAA CCT ATC GGT TA-5'
5'-TTT GGA TAG CCA AT-3'
N-Phe Gly Stop

Promotion Stage

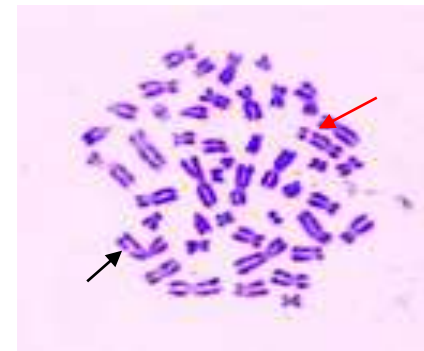
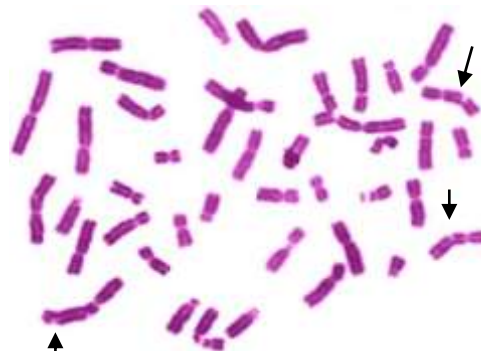
- second stage of the carcinogenesis process, derived from either **endogenous or exogenous stimuli of cell growth (promoters)**
- promoters are **not mutagenic** and not able to induce tumors by themselves; rather they act through several mechanisms:
 - **gene expression changes** that result in sustained **cell proliferation** either through increases in cell proliferation and/inhibition of apoptosis
 - acting through **receptor or non-receptor mediated** processes
- clonal expansion of initiated cells to **produce a preneoplastic lesion**, which requires repeated or continuous exposure to promoters
- **threshold effects** - below a certain dose or frequency of exposure, tumor promoters are unable to induce initiated cell proliferation
- promoters show **organ-specific** effect (e.g. phenobarbital - liver)
- mutation in regulatory region of **telomerase gene** – continuous expression
- **reversible** stage
- most usually takes more than 5 years

Progression Stage

- third (final) stage of the carcinogenesis process
- **conversion** of the benign preneoplastic lesions **into a neoplastic cancer**
 - result of occurrence of genotoxic events inducing **chromosomal** damage such as **aberrations and translocations**
 - mediated by **increase in DNA synthesis and cell proliferation** in the preneoplastic lesions (promotion stage)

Chromosome aberrations: breaks and structural changes.

Breaks: - occur in S phase of cell cycle when primary damage hinder DNA replication by inhibiting topoisomerase II
- in G₂ phase by interaction of DNA with free radicals

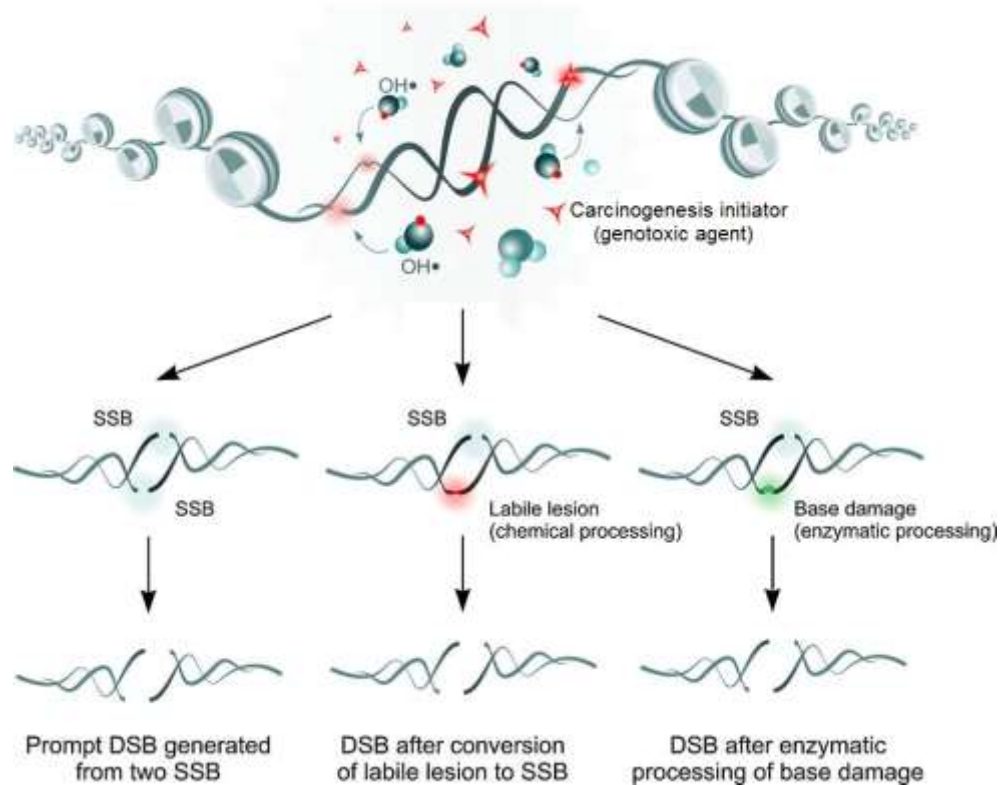


—→ **Chromatid break**

—→ **Chromosome break**

Chromosome structural changes:

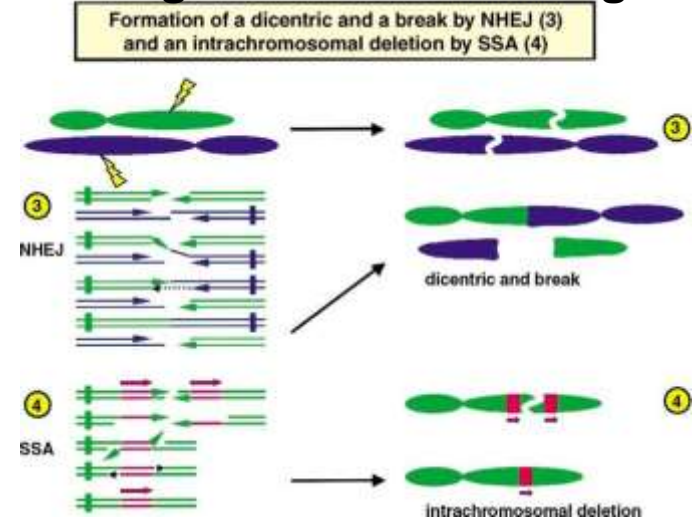
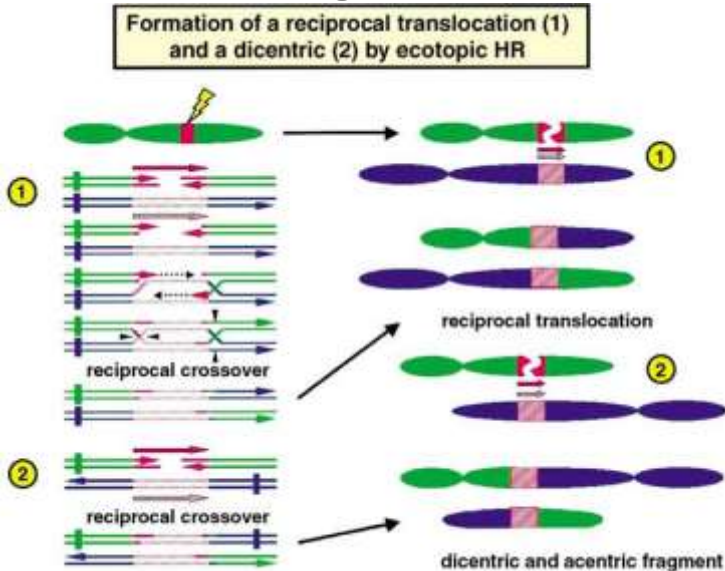
In **G₁ phase single strand breaks** should be transduced into **double DNA strand breaks** by different mechanisms



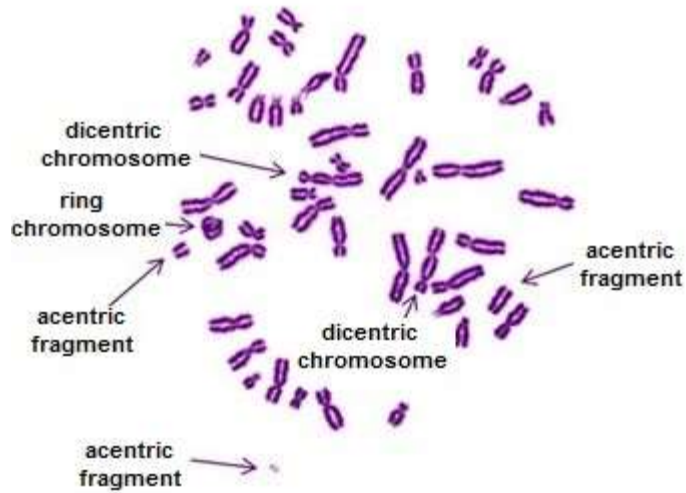
Chromosome structural changes:

- different **error-prone repair** mechanism misrepair double DNA strand **breaks** forming:
 - acentric fragments,
 - dicentric chromosomes,
 - multicentric chromosomes
 - deletions
 - translocations

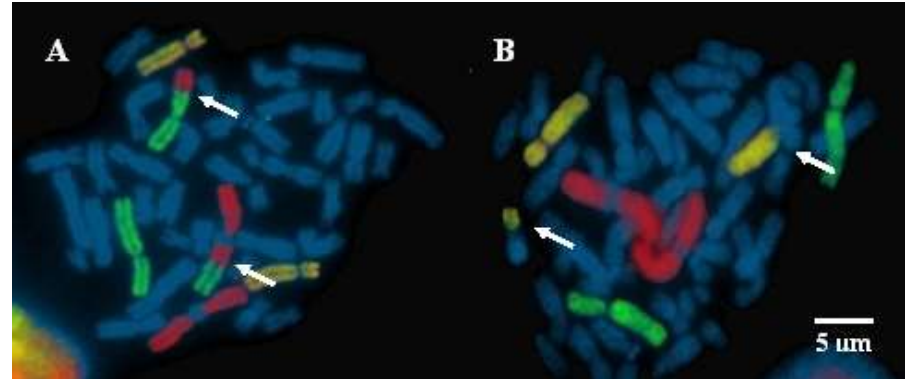
Ectopic homologous recombination (EHR), Nonhomologous end joining & Single-strand annealing



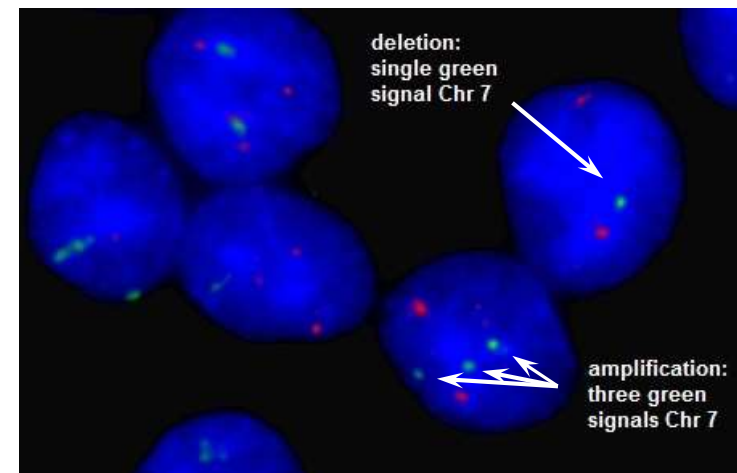
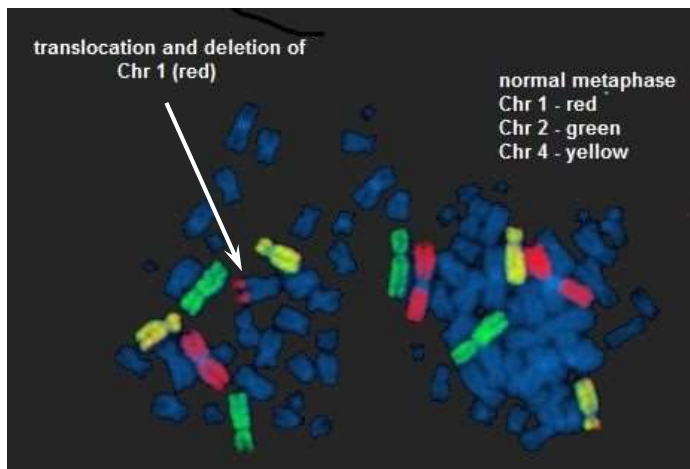
Structural chromosomal aberrations



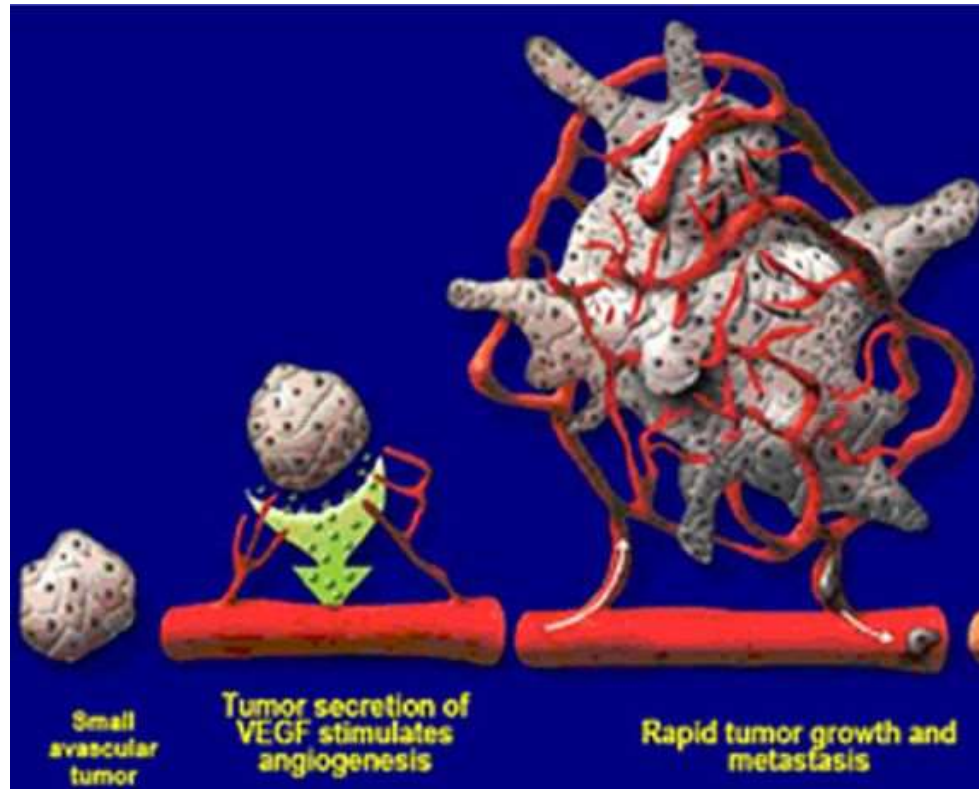
Translocations



Deletions and amplifications



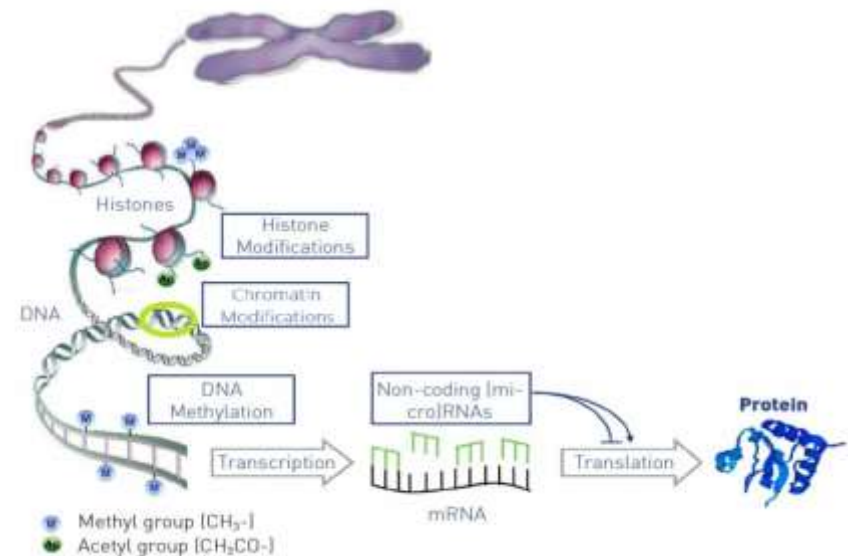
- **accumulate a variety of mutations** and epigenetic changes that cause them to outgrow the surrounding cells (hypermutable stage)
- **neoangiogenesis** - extreme **increase in expression *ras* oncogene** (mutation or hypomethylation of promoter region, deacetylation, gene amplification, translocation near strong promoter region) results in synthesis and release of **proangiogenic factors** VEGF, FGF, PDGF - enables increased supply with **nutrients, oxygen**; enables **invasiveness and metastasis**



- **clonal nature** of the neoplastic lesion is being **lost**
- lack of cell cycle and growth control, DNA repair (**mutator phenotype**)
- **irreversible stage**
- accumulation of **nonrandom chromosomal aberrations** and karyotype instability are **hallmarks of progression**

Epigenetic (Nongenotoxic) Carcinogenesis

- **epigenetic (nongenotoxic) carcinogens** is induced indirectly, by agents that do **not get in contact (do not interact) with DNA**, but may:
 - induce **production of endogenous chemical species** that do damage DNA
 - themselves **interact with other proteins** involved in **DNA physiology** →
 - **altering genes expression**
 - promotor CpG (de)methylation
 - histone (de)acetylation
 - histone Ser (de)phosphorylation
 - miRNA synthesis alteration
 - chromatin remodeling
 - **hindering DNA repair**
 - **apoptosis inhibition**
 - **DNA repair enzymes inhibition**
 - **interfering with replication protein.**
 - **oxidative stress induction**
 - **interfering with intracellular signaling pathways**



Main promoters of the epigenetic carcinogenesis are:

- long-term **oxidative stress** (induces DNA damage)
- stimulation of **progression of cell divisions** (fixation of primary damage as mutations or chromosomal aberrations; exhaustion of repair processes = less efficient)

- effect is **reversible**
- there is a **threshold**

- frequently epigenetic carcinogens **functions** at the **tumor promotion stage**
- most **epigenetic carcinogens** are promoters, **not initiators**

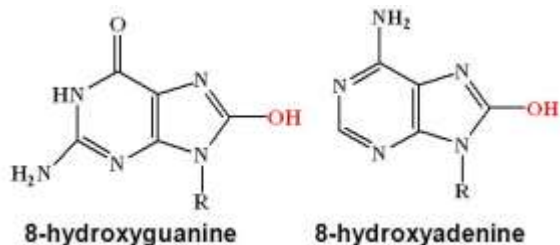
Oxygen and Nitrogen Species as Cancerogenesis Inducers

ROS – Reactive Oxygen Species

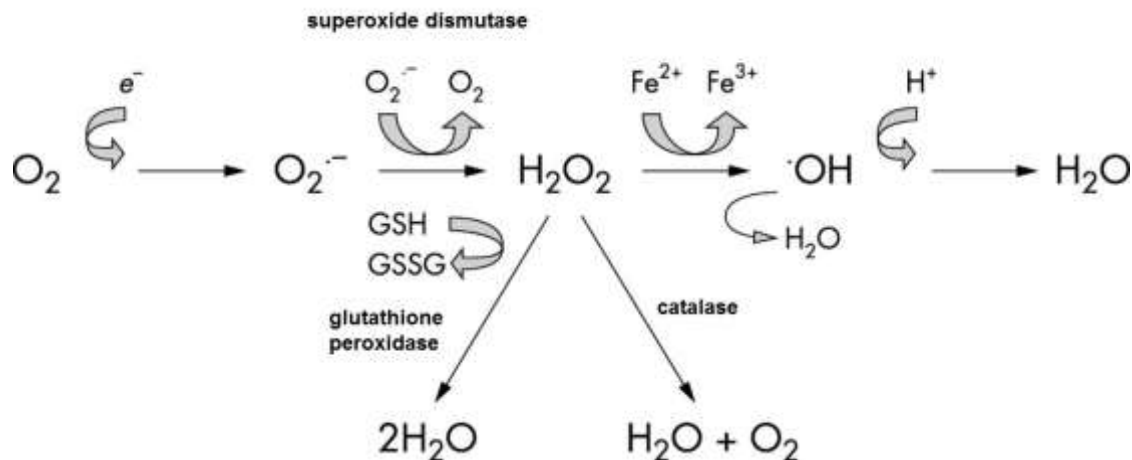
- formed endogenously – cellular metabolism

| | | | |
|------------------------|-----------------------|-----------------------|------------------------|
| H_2O_2 | $\text{O}_2^{\cdot-}$ | HO_2^{\cdot} | OH^{\cdot} |
| Hydrogen peroxide | Peroxide ion | Perhydroxyl radical | Hydroxyl radical |
| O | $\text{O}^{\cdot-}$ | O^{2-} | $\text{O}_2^{\cdot-}$ |
| Singlet oxygen | Oxene ion | Oxide ion | Superoxide radical ion |

Oxidatively changed DNA bases:

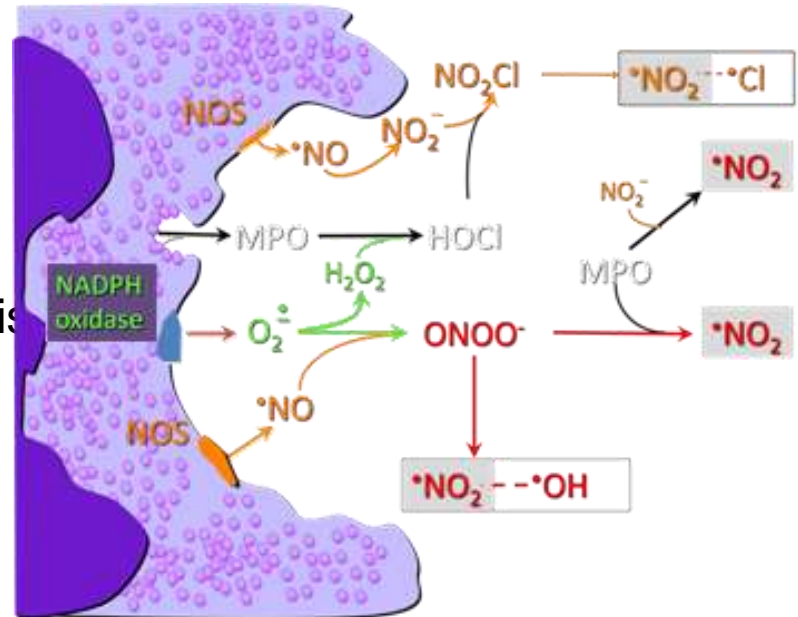


Pathways of ROS Neutralization



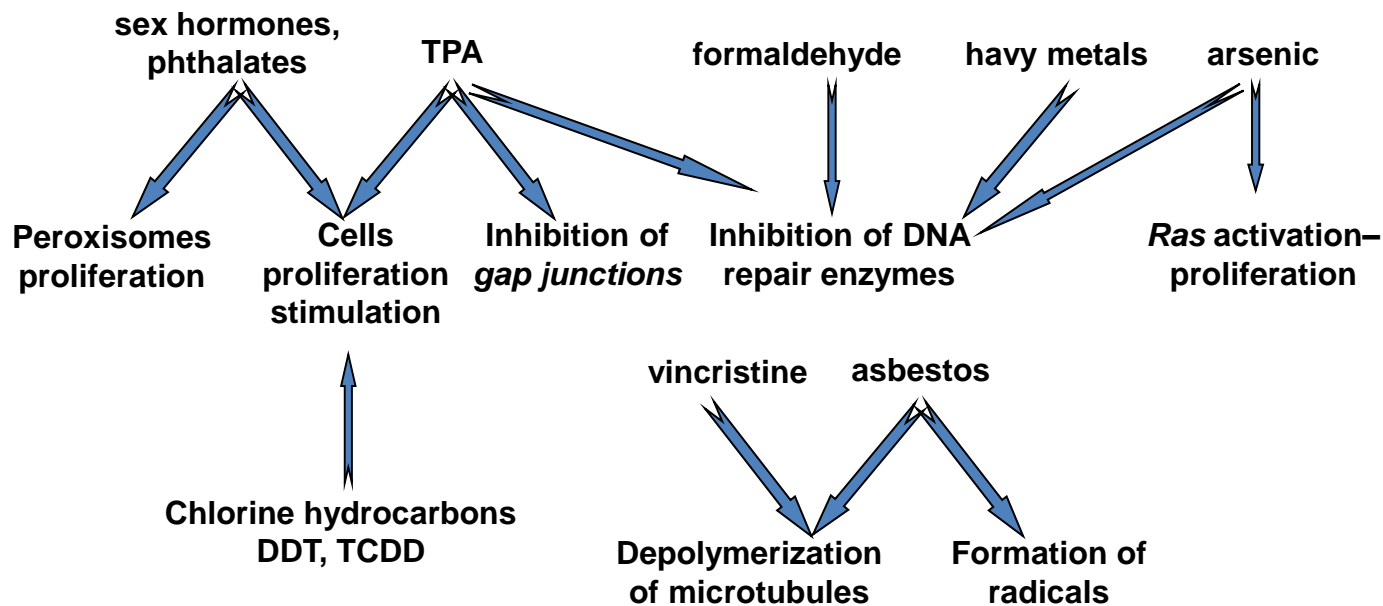
Reactive Nitrogen Species (RNS)

- NOS = **Nitrogen Oxide Synthase** utilises L-Arg to synthetise **NO**
- NO = **signaling molecule** – angiogenesis, vasodilation, erection, peristalsis, insulin secretion, memorizing, developement of nervous system
- MPO = **MyeloPerOxidase** synthetizes **hypochlorite** from H_2O_2 and transduces **peroxynitrite $ONOO^*$** into **nitrite radical** (nitrogen dioxide radical *NO_2); besides, it synthetizes them out of nitrite ions
- Beside being DNA reactive by themselves, **$ONOO^*$** dissociates to form ***OH** (ROS) and **NO_2Cl** to form ***Cl**
- **RNSs affect proteins** involved in DNA replication (DNA polymerase), repair, protein kinases (JNK1 by *NO - apoptosis inhibition)



Other examples of promoters:

- tetradecanoylphorbol acetate (**TPA**) – activates protein kinase C which stimulates cell division
- **Cd** and **Ni** inhibit methyltransferase DNMT1 and other DNA repair enzyme
- **As** - hypomethylation of *Ras* promoter region
- **asbestos** and **wood dust** (possibly nanoparticles) highly induce formation of ROS and RNS in macrophages → continuously damages surrounding tissue → a need for their replacement by promotion of division of pluripotent mesenchymal cells → s Phase mutation occurrence = **bystander effect**)



Carcinogens Classification in EU

IARC (International Agency for Research on Cancer)

| Classification (number of agents classified as) | Name | Meaning | Example given |
|---|---------------------------------|--|---|
| Group 1 (118) | Carcinogenic to humans | sufficient evidences of carcinogenicity in humans EXEPTIONALLY sufficient evidences in experimental animals and strong evidence in exposed humans that it acts through carcinogenic mechanism | aflatoxin asbestos 4-aminobiphenyl |
| Group 2A (79) | Probably carcinogenic to humans | limited evidences of carc in humans and sufficient evidences in exp animals and that it is mediated by mechanisms that operate in humans EXEPTIONALLY only limited evidences of carc in humans | acrylamide adriamycin <i>N</i> -methyl- <i>N</i> -nitrosourea |
| Group 2B (290) | Possibly carcinogenic to humans | limited evidences of carc in humans and less than or sufficient evidences in exp animals EXEPTIONALLY inadequate evidences of carc in humans and less than sufficient in exp animals | acetamide bitumen caffeic acid |

| Classification (number of agents classified as) | Name | Meaning | Example given |
|---|--|--|-------------------------------|
| Group 3 (501) | Not classifiable as to its carcinogenicity to humans | inadequate evidence in humans and inadequate or limited in experimental animals EXCEPTIONALLY inadequate in humans and sufficient in exp animals with strong evidence that mechanisms of carc do not operate in humans | melamine phenol toluene |
| Group 4 (1) | Probably not carcinogenic to humans | evidence suggesting there is a lack of carc in humans and exp animals OR Inadequate evidences in humans but evidences suggesting lack of carc in exp animals | caprolactam |

carc - carcinogenicity/carcinogenic

exp - experimental

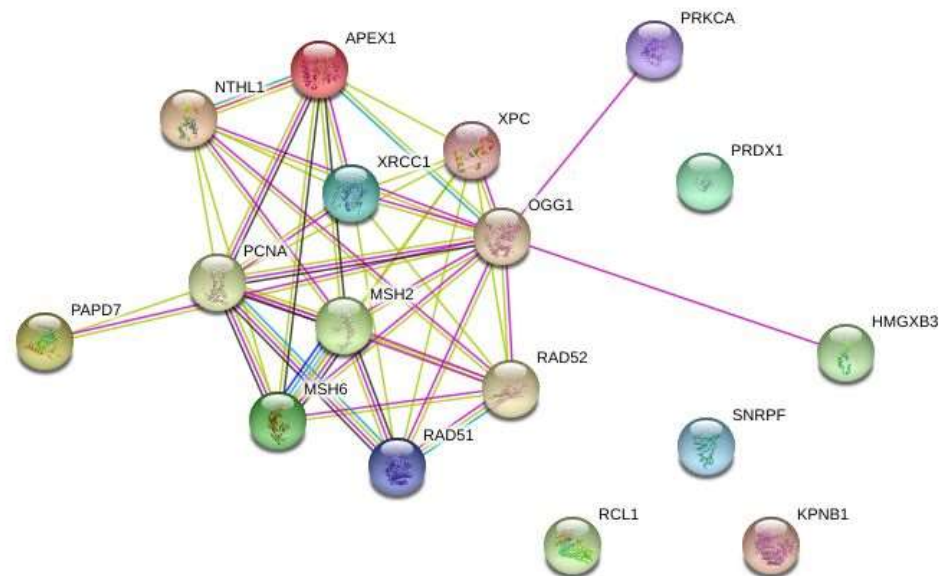
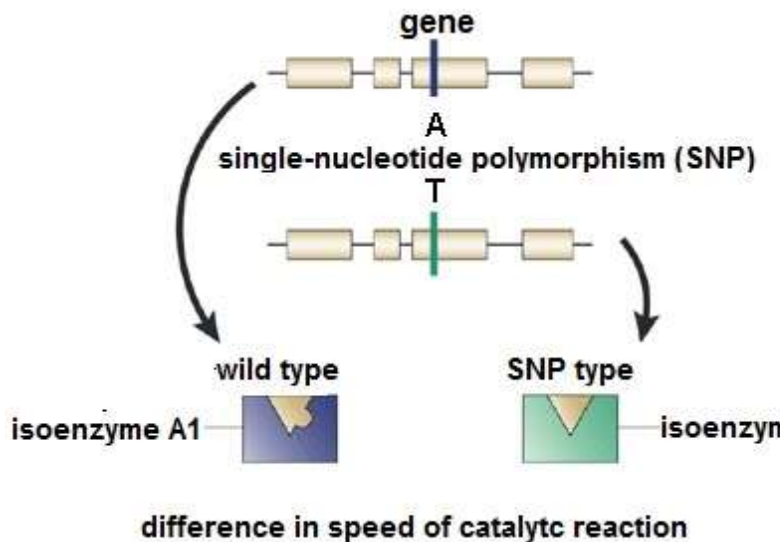
<http://monographs.iarc.fr/ENG/Preamble/CurrentPreamble.pdf>

Single Nucleotide Polymorphism (SNP) of the Genes in Carcinogenesis

Some discrepancies:

1. More than 80% of bronchial (lung) carcinoma patients = cigarette smokers
less than 15% “heavy” cigarette smokers = bronchial carcinoma patients
2. 66% urinary bladder carcinoma patients = cigarette smokers
less than 10% cigarette smokers = urinary bladder carcinoma patients
3. More than 90% gastric carcinoma patients = infected by *Helicobacter pylori*
5 % infected by *Helicobacter pylori* = gastric carcinoma patients

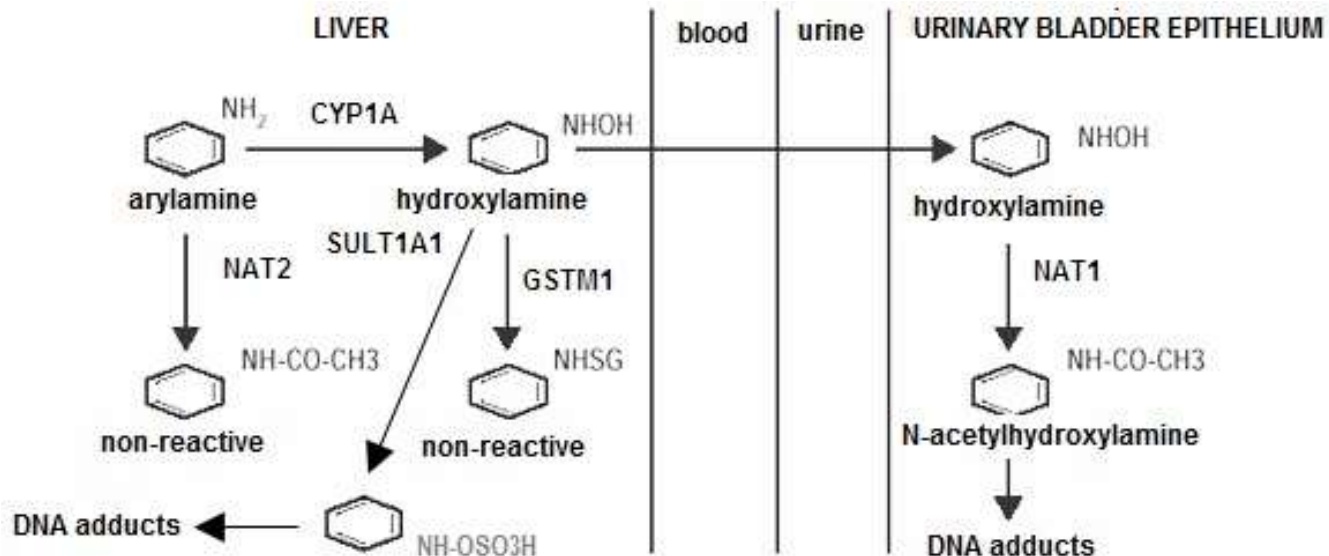
- in human population there are more different alleles of the same gene or genetic locus
 - Result of point mutation (conservative missense mutation) = *Single-Nucleotide Polymorphism (SNP)* → different amino acid but protein retains its tertiary and quaternary structure and function = isoenzymes
 - SNPs of two gene groups affect carcinogenesis
 - genes encoding enzymes of 1. and 2. phase of metabolic transformation
 - DNA repair genes
- leading to
- differences in the velocity of metabolic reaction and repair efficiency
 - elevated/reduced risk of developing a neoplasm



NAT1/NAT2

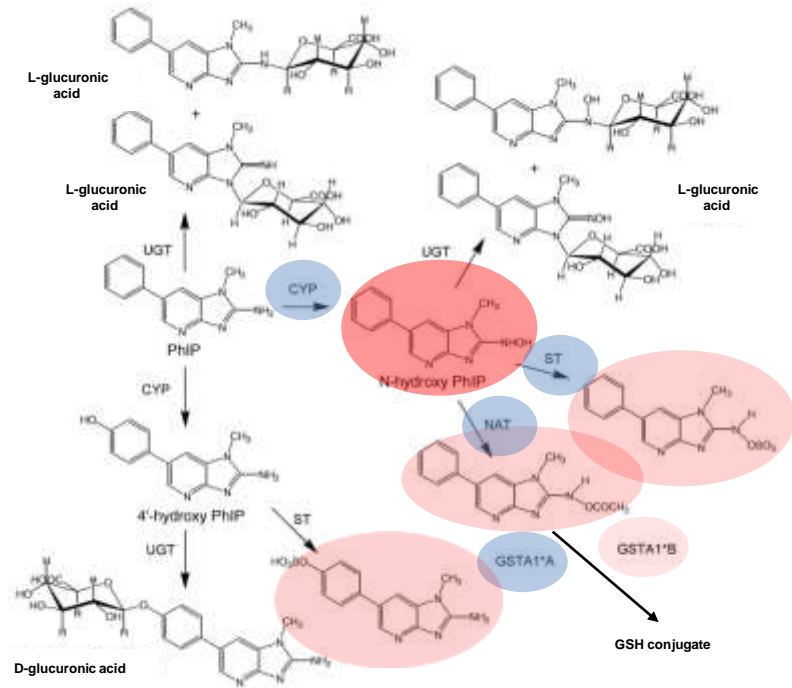
(NAT – N-acetyltransferase)

- N-acetyltransferase → N-acetylation of arylamine (4-aminobiphenyl - cigarette smoke)
- NAT2 isoenzymes (SNP) - NAT2*4 10x lower catalytic activity than NAT2*7 (liver and small intestine)
- NAT2*4 – higher risk from urinary bladder carcinoma in cigarette smokers and occupational exposure



GSTM

- glutathione S-transferase
- GSTM0 (non-active gene – both alleles), GSTM01 (non-active only 1 allele), GSTM1 (both alleles active)
- Urinary bladder carcinoma (50-70% higher risk for GSTM0) – regardless of smoking habits



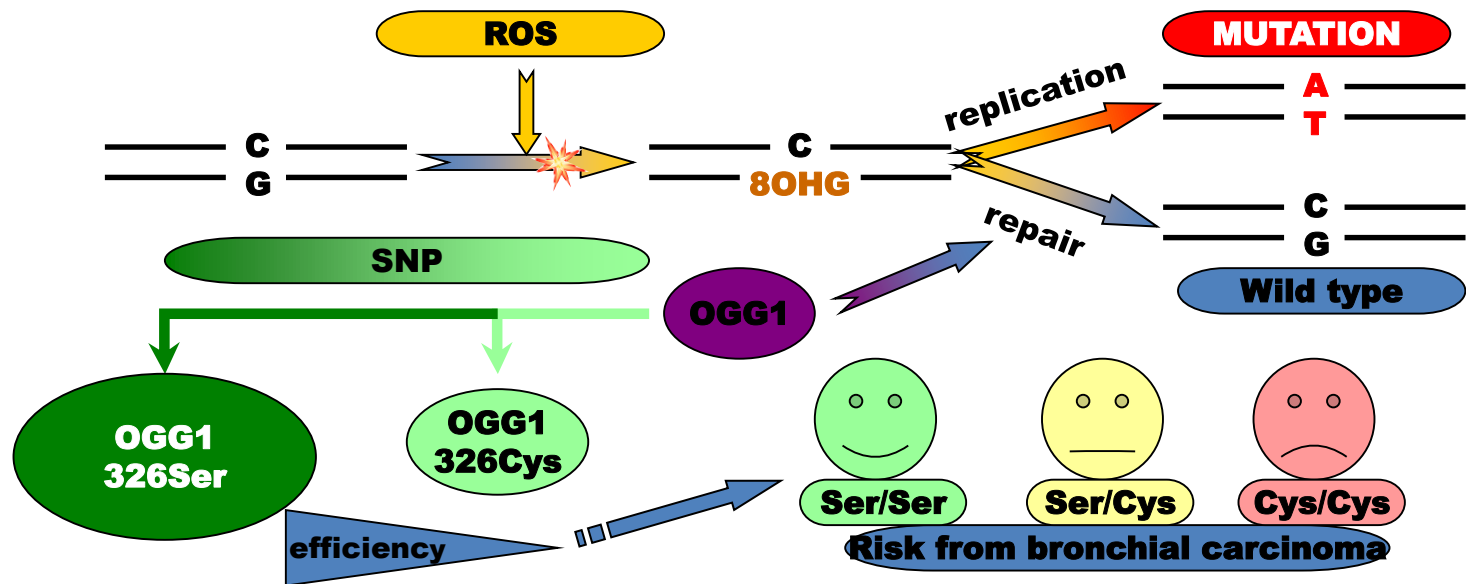
CYP and NAT

- N-hydroxylation
- CYP1A2 – highly catalytic isoenzyme
- Heterocyclic amines – thermal processing of red meat
- Colon carcinoma (higher risk CYP1A2 + NAT2*4) – N-hydroxy and N-sulfoxy metabolites direct (primary) mutagens

OGG1

8-hydroxydeoxyguanine glycosylase

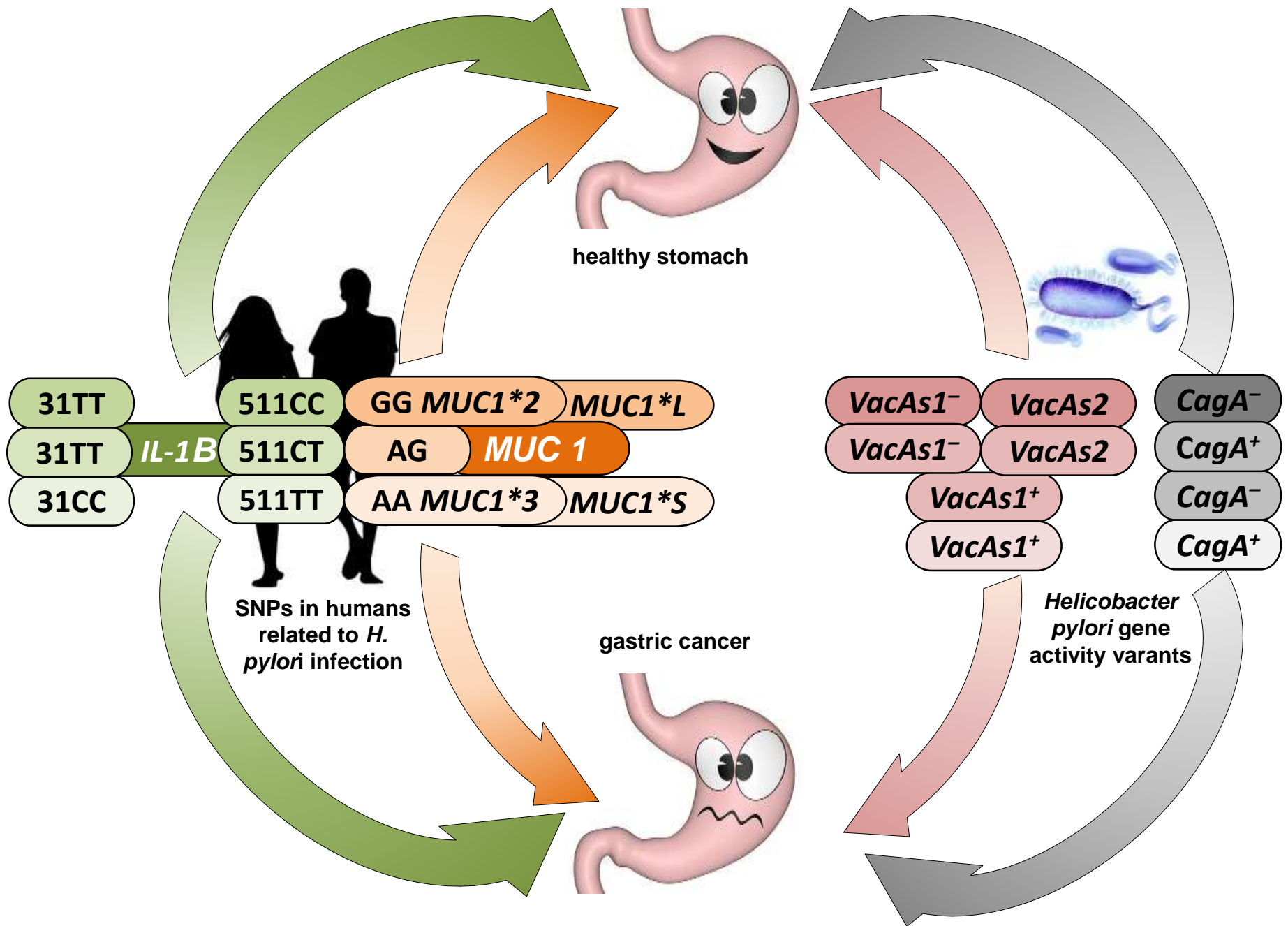
- repair of oxidized nitrogenous bases (mostly 8OHG) - BER



Codon degeneracy:

Ser: UCU or UCC or UCA or UCG

Cys: UGU or UGC



Carcinogenicity testing

- testing takes ≥ 1 year
- expensive
- tumor formation rather rare event: increase in occurrence from 2 to 6%;
- opposite – hepatocellular carcinomas in rats pretty often event – difficult to distinguish spontaneous from the induced one
- to minimize possibility of false-negative results:
 - large number of animals (minimum 50/gender/dose(group); 3 doses minimum)
 - chronic administration (2 years)
 - high doses (to induce carcinogenesis), relatively short exposure period - does not reflect real situation (except accidental events) → may cause:
 - inflammatory processes → epigenetic carcinogenesis \neq (in)direct chemical initiator
 - maximum tolerable doses (MTD) had have been used; recently recommended by ICH: Tested dose has to reflect the one used in therapy: calculated ratio of dose and blood volume of rat and human x 25
 - but, suspected genotoxins should continue to be tested at MTDs

Other possible issues:

- **high dose** testing → **overload** of metabolic enzymes
- **mortality** in a control group should be <10%, in a treated one <50%
- “searching for a needle in a haystack” – **biopsy of all organs and tissues in all animals**
- issue and **imperfection of extrapolation** of the results to humans (e.g. artificial sweeteners aspartame and saccharine)

Thus, additional **surrogate carcinogenicity tests** are performed (genotoxicity and mutagenicity tests):

- ***in vitro***:
 - Ames test
 - chromosomal aberration test
 - cytokinesis-blocked micronucleus test
- ***in vivo***:
 - transgenic rodent gene mutation assay (TGR)
 - bone marrow chromosomal aberration test
 - bone marrow / reticulocytes (in mice only) micronucleus test

Medium-term (shorter-lasting) carcinogenicity tests

- transgenic animals:
 - **inserted activated oncogenes** (TgHras2)
 - **knocked-out** one or both **tumor-suppressor** gene copy(ies) (p53^{+/-} or p53^{-/-}),
 - **knocked-out** one or both **DNA repair enzyme** gene copy(ies) (XPA^{+/-} or XPA^{-/-})
- **higher susceptibility** to carcinogens, shorter initiation and promotion stages
- **shorter time of administration** of the tested chemical - 6 months

Shortcomings:

- possible **false-negative** results due to **inappropriate strain choice** – p53 is unimportant in development of hepatocellular carcinoma → p53^{+/-} strain = false-negatives for aflatoxin
- **genes modified** in chosen transgenic animal strains may **not be important** for **carcinogenesis pathway** of a tested chemical