Considerations on the carcinogenicity of Ethane-1,2 diylbis[nitrilobis (methylene)] tetrakisphosphonic acid (EDTMP)

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EDTMP mode of action and mechanisms of toxicity

EDTMP is a metal cation chelator that can bind iron and remove it from transferrin, leading to iron cleavage and facilitating urinary iron excretion (Calvin et al., 1988). High doses of EDTMP (up to 500 mg/kg body weight) administered orally to rats induced anemia by enhancing iron excretion, preventing iron utilization, and depleting iron stores. Lower doses (up to 100 mg/kg/b.wt.) had no effect on iron absorption. EDTMP toxicity is influenced by dose and route of administration, with subcutaneous administration at low doses (37 mg/kg) being highly toxic and decreasing animal body weight, affecting intestinal calcium absorption, increasing calcium plasma levels, and decreasing bone growth. When administered by gavage for 7, 28, and 90 days, bone changes were observed only at the highest tested dose of 333 mg/kg/day. wt. Administration of EDTMP for 13 weeks did not affect calcium homeostasis up to a dose of 500 mg/kg. wt. The lower toxicity of EDTMP in dietary studies is likely due to reduced absorption in the gastrointestinal tract. After gavage administration, almost 50% of the chemical is accumulated in the femur (Calvin et al, 1988).

Carcinogenesis bioassays for EDTMP

Four carcinogenicity studies were performed using EDMP. Three studies were performed on rats. One study was performed using mice.

Study #1
Carcinogenesis study of EDTMP-H-Na *(Cas 244-742-5)* by gavage in female and male Sprague-Dawley rats (Study Report 1985)

Tested doses (mg/kg/b.wt)	Incidence of osteosarcomas		Historical controls for SD rats (	
	Males	Females	Male	Female
0	0/60	0/60	1.11-2.67 %.	2.67%
15	0/60	0/60		
50	1/60 (0.2%)	0/60		
150/333	28/60 (47%)	4/60 (7%)		

Study #2
Carcinogenesis study of EDTMP-Na *(Cas 244-742-5)* in female and male Fisher 344 rats by oral administration (Calvin et al, 1988)

Tested doses (mg/kg/b.wt)	Incidence of combined pancreatic islet cell adenomas and carcinomas in female rats			Historical controls for female F344 rats
	Adenomas	Carcinoma	Combined	
0	0/50	0/50	0/50	4.4 -5.4%
4	2/50	0/50	2/50	
20	2/50	1/50	3/50	

100	4/50	1/50	5/50	

Study #3
Carcinogenesis study of a mixture EDTMP-Na *(Cas 244-742-5)* and sodium fluoride (NaF) by gavage in female and male Sprague-Dawley rats (Study Report 1986c)

Tested doses EDTMP (mg/kg/b.wt)	Tested doses of NaF (mg/kg/b.wt)	Incidence of osteosarcomas		Historical controls for SD rats (	
		Males	Females	Male	Female
0	0	0/45	0/45	1.11-2.67 %.	2.67%
15	1.139	0/40	0/40		
75	5.695	15/40 (37%)	11/40 (27%)		
150	11.390	24/40 (60%)	11/40 (27%)		

Study #4
Carcinogenesis study of EDTMP-H-Na *(Cas 244-742-5)* by gavage in female and male B6C3F1 mice (Study report, 1986d)

Tested doses (mg/kg/b.wt)	Animals at risk		Carcinogenic effects
	Males	Females	
0	85	85	None
15	85	85	None
75	85	85	None

The first study (Study #1) was performed on Sprague-Dawley rats in 1985. Animals were treated with the chemical (purity 97 %) at doses of 0, 15, 50, and 150 mg/kg body weight (60 animals/treatment), by gavage. At day 329 (week 47), the top dose was increased to 333 mg/kg body weight

Both males (28/60, 47%) and females (4/60, 7%) developed osteosarcomas at the highest tested dose.

Even if the study reflects the OECD guidelines for rodent carcinogenicity bioassays, the experimental design is constrained by the limited knowledge available at the time, resulting in certain limitations.

In this study, one male animal (1/60, 0.2%) in the middle treatment group developed tumors. This result should not be considered significant because the incidence was below the osteosarcoma incidence in the historical controls for this species and strain. The use of historical controls is of paramount importance during the process of analyzing data from a carcinogenicity study, since relying solely on a positive statistical difference as evidence of a true effect in treated group compared to a concurrent control group, could lead to a false positive result. Additionally, a slight increase in the incidence of a rare type of cancer may not be statistically significant enough to be detected by typical tests used in toxicology studies. In such cases, historical control data can be used to justify the biological significance of a small increase in the incidence of uncommon cancers. However, based on historical controls from 31 studies, the incidence of osteosarcoma in Sprague-Dawley rats was the range–1.11-2.67 %, which is coherent with the observation that osteosarcoma is the most common bone tumor in rat as in human.

Therefore, cancer develops only at the top doses, which probably exceed the maximum tolerated dose, without showing any dose-effect relationship.

The highest dose was increased based on the level of alkaline phosphatase. Serum alkaline phosphatase is used as a marker of cancer spanning the bones and liver and is considered a valuable tumor marker with high specificity in human osteosarcoma. However, the serum levels of alkaline phosphatase did not change throughout the reported experiment, even in animals affected by osteosarcoma.

The lack of variation in alkaline phosphatase led to doubling the top dose at week 47, supposedly with the aim of triggering the carcinogenesis process. However, the first palpable bone tumor was registered in one male animal at week 35 and in female animals at week 45, suggesting that treatment variation affected the incidence and progression of the disease. Animal were treated with a dose, 333 mg/kg body weight, that affects calcium homeostasis, induces bone changes, decreases body weight and induce iron depletion (Calvin et al, 1988).

Based on the results from subchronic studies (Miller et al. 1985), the top dose used in this study can be considered the maximum tolerated dose (MTD).

MTD is the highest dose of a substance that can be tested in long-term carcinogenicity studies as stipulated by regulatory authorities. It is a limit range, which was intentionally selected for toxicological evaluations to ensure that all potential carcinogenic effects would be thoroughly examined, including those that might not have been detected due to statistical limitations commonly encountered in small animal cohorts used in such studies. However, there is growing concern regarding MTD findings because non-specific high-dose effects may lead to misleading "positive" responses that are not indicative of lower exposure levels.

In the Study #1, a marked depression of erythrocyte count, hemoglobin concentration and hematocrit value were reported in animals treated with the highest dose by month 6, weeks before the appearance of the first palpable lesion. The altered parameters could be the indicator of iron-deficiency anemia, that was described at dose exceeding 100 mg/kg body weight.

Iron plays a crucial role in cellular activities due to its unique ability to exchange electrons, and metabolic abnormalities of iron can lead to a range of diseases. The body carefully regulates the amount of iron in the system and cells to prevent iron deficiency or overload from causing harm.

Some research has found a connection between iron metabolism and the development of osteosarcoma. In osteosarcoma, the pathways and molecules involved in iron import and storage are activated, while those involved in iron export are restricted and inhibited. It has been reported that an excessive iron can promote osteosarcoma occurrence.

Iron is depleted from cellular compartments through the chelating action of EDTMP, and transported to the longitudinal bones where it accumulates. This accumulation is sustained and exacerbated by the increasing chemical treatment, thereby creating an environment conducive to the development of osteosarcoma.

Consequently, the development of osteosarcoma may be directly attributed to the elevated levels of iron delivered to the longitudinal bones by EDTM, a phenomenon that only occurs at high concentrations of EDTM and is not relevant at the levels of human exposure.

The potential carcinogenic effects and other related effects that are not significant at low doses may warrant reassessment of the classification of a substance. The decision to not classify a substance should be based on the mechanism of action that is effective at high doses but not evident at lower levels of exposure, even in animal studies.

In conclusion, the Study #1 cannot be considered for the classification of EDTMP as a carcinogen due to the following reasons:

- The cancer-causing effects are directly related to toxic behavior of EDTMP that has only been observed at high doses.
- There is no observable dose-response relationship.
- No other tumors have been reported in any other site

In Study #2, the compound induced tumors in the pancreatic islets. The authors believe that the results of this study should not be considered due to the extremely low incidence in the controls compared to historical controls.

Study #3 was conducted with a mixture of EDTMP and sodium fluoride. The results are compromised by the high mortality of animals during the treatment period at the two highest doses tested, leading to a significantly reduced number of surviving animals. It is therefore believed that the tumors obtained at these doses are a consequence of excessive toxicity, The administration of substances at doses approaching the maximum tolerated dose (MTD) is believed to stimulate regenerative growth, thereby increasing the likelihood of mutagenicity and, consequently, secondary carcinogenicity induced by toxic regenerative response.

Based on these considerations, the chemical cannot be classified as a carcinogen, as supported by the available studies, including a negative report in mice, which is considered a more suitable animal model to represent human osteosarcoma.

Human relevance of animal results:

It has been reported that the chemical-induced model of osteosarcoma might not resemble the biology of human tumors because the treatment disrupts the microenvironment, which plays a key role in the maintenance of tissue homeostasis and whose perturbation plays the main role in cancer progression (Pu et al, 2023). Other considerations should be taken into account to evaluate the human relevance of animal results.

1) **incidence and prevalence of osteosarcoma in human and animals**

Osteosarcoma is a type of cancer that is characterized by the presence of malignant cells that originate in the bone marrow. Histologically, osteosarcoma is classified into three major subtypes: osteoblastic, fibroblastic, and chondroblastic, with osteoblastic being the most common.

The incidence of this tumor in the general population is approximately 2-3 cases per million per year, and is more common in adolescence, with an annual incidence peak of 8-11 cases per million per year in this age group. Among adolescents aged 10-19 years, osteosarcoma represents 15% of all extracranial tumors and is 1.4 times more frequent in men than in women.

Based on the European Union definition of rare diseases and common cancers, rare cancers are considered to have an incidence of less than six cases per 100,000 individuals per year (0.006%). Primary bone sarcomas are infrequent cancers, accounting for approximately 0.2% of all malignant tumors. More than 90% of primary bone sarcomas are classified as osteosarcomas. Osteosarcoma is a rare disorder, but it is the most common cause of bone cancer in children and adolescents.

Historically, bone or joint lesions caused by both spontaneous and induced factors have been thought to be relatively uncommon in rodents. However, there has been an increasing trend in the use of chemical and pharmaceutical agents that can cause morphological changes in bones and joints, which are typically detected in routine toxicity assessments.

Accumulating evidence shows marked differences between species, strains, and sexes in the development of spontaneous primary bone sarcomas.

Rats are susceptible to osteosarcoma (OS), one of the most common malignant bone tumors in this species. Males are more likely to develop OS than females, with a ratio of four one. Most OSs in rats develop in the appendicular skeleton, although they can also arise in soft tissue outside the skeleton, known as extraskeletal OS (ESOS), which originates in the subcutaneous tissue, spleen, and digestive tract.

2) Natural history of osteosarcoma and factors affecting the appearance and progression of the disease in humans

a. Age

The incidence of osteosarcoma is highest during periods of skeletal growth, particularly in the first decade of life, and it occurs again in the geriatric population. In children, adolescents, and young adults, the majority of cases are high-grade and begin in the intramedullary space of metaphyseal locations in long bones of the lower extremity, which suggests a relationship with active growth plates. The incidence of osteosarcoma is low in individuals between 25 and 59 years of age, but it rises again in individuals over 60 years of age, and is most often associated with Paget’s disease or radiation exposure. This implies that the fundamental cause of the disease might not be the same in young and older individuals.

b. Bone growth and tumorigenesis

Osteosarcoma tends to occur in rapidly growing bone, specifically around the knee, which is supported by numerous studies that have established a correlation between rapid bone growth during puberty and osteosarcoma development. The epiphyseal growth plates (GP) of the distal femur and proximal tibia are responsible for much of the increase in height that occurs during puberty. Moreover, the peak age of osteosarcoma development is slightly earlier for females, which may be attributed to the earlier growth spurt experienced by girls during puberty. Individuals affected by the disease tend to be taller than the normal population of the same age group. Additionally, patients with Paget's disease, a condition characterized by excessive bone formation and breakdown, have a higher likelihood of developing osteosarcoma.

c. Hormonal status

The involvement of hormones in bone metabolism is well-established. Several hormones have been extensively studied in this context, including growth hormone,

prolactin, somatomedin, growth factor similar to insulin, parathormone, thyroid hormones, and steroid hormones. The latter three play a crucial role in maintaining the integrity of bone structure. Osteoblasts are capable of producing biologically functional estrogens and androgens from circulating steroids. Several studies have suggested the possible role of sex hormones in the pathogenesis of osteosarcoma in long bones, especially in adolescent males. Indeed, research has established that androgens promote osteoblastic differentiation, and androgen receptors (AR) have been detected in osteosarcoma, with higher abundance in metastatic osteosarcoma. Moreover, the epigenetic silencing of the estrogen receptor is a characteristic feature of human osteosarcoma, and the application of 17-beta estradiol has been demonstrated to promote differentiation in osteosarcoma cells (Manolaga et al., 2013).

3) Limitations of rodent carcinogenicity bioassay to replicate human disease

a. Age

Animal age is one of the most critical factors in the extrapolation of experimental results to humans.

Rodents have a shorter and more rapid early lifespan. Therefore, their maturation rate in the first month of life is 150 times faster than that of humans, and it continues to accelerate at a rate of 45 times between 1 and 6 months and 24 times when they reach 6 months of age. Rats and mice have similar developmental profiles and reach sexual maturity around day 21 after separation from their mother. In females, sexual maturity occurs at day 32-34, while in males, it occurs at day 45-48. Sexual maturity marks the beginning of adolescence, which lasts until week eight after birth. Although body weight can be used as an indicator of age, it is an imprecise marker. Rats were considered young adults at 63 days or 1.5 to 2 months, depending on their weight (230 g/300 g). However, rats of the same age can vary in weight by up to 100 g, which can result in a difference of 3 weeks in age from day 49 (periadolescent) to day 70 (young adult). According to OECD TG 451, animal treatment should begin as soon as possible after weaning and before eight weeks of age. Therefore, the experimental groups included animals at different levels of development. The experimental groups may include animals at various stages of early development, ranging from 12.5 to 14 years of age for humans (still considered juveniles, not adults). Female reproductive senescence (menopause) and overall senescence (senescence biomarkers) occur earlier in rats than in humans. Thus, treatment starts during the periadolescent period and continues until the animals reach (or may have passed) reproductive senescence. This study did not cover prenatal exposure, postnatal exposure, development until sexual maturity, and later senescence. (Paparella et al. 2017)

In conclusion, the rodent carcinogenicity bioassay cannot correctly replicate the age windows that are critical for human disease.

b. Bone growth and tumorigenesis

Bone development in rodents, especially rats, shows several species-specific characteristics related to age. The physiology and timing of the closure of the growth plate (physis) vary among bones and species, and it is important to consider these differences when selecting a research model involving bone growth. The choice of model should consider the age and status of sexual maturity at the time of growth plate closure.. It is assumed that bone length growth is complete when sexually mature animals are chosen for experimentation, as most mammals experience cessation of bone growth shortly after sexual maturity. However, growth plates in rats have been reported to remain open for extended periods of time, even after sexual maturity, and throughout the normal life expectancy of the animal, which makes it inappropriate to study bone diseases in this species (Bagi et al, 2011)

c. *Hormonal status*

Based on the aforementioned factors, it is essential to consider the hormonal status of the animals used in the carcinogenesis bioassay, as it may impact the final outcome. Therefore, a thorough assessment of hormonal status should be conducted across the dataset, with particular attention paid to sensitive endpoints in peripubertal age (Paparella et al, 2017)

4) Animal models in osteosarcoma

Currently, there is no animal model that can accurately replicate the natural progression of human disease.

The spontaneous tumor model of canine osteosarcoma closely resembles that of human osteosarcoma in terms of biological characteristics. However, osteosarcoma develops in skeletally mature geriatric dogs, which is different from humans, where the peak of incidence occurs during adolescence (Guijarro et al, 2014).

Several mouse models have been established to replicate the key molecular events, such as, P53 and RB mutations, present in both hereditary and sporadic forms of human osteosarcoma. However, the rapid development and higher incidence of other tumors (mostly lymphomas), and the long latency of osteosarcoma necessitate the sacrifice of the mice before osteosarcoma onset, hampering in many cases the utility of these models (Guijarro et al, 2014).)

Environmental factors playing a role in osteosarcoma

Several agents, including physical, chemical, and biological factors, have been proposed as potential causes of osteosarcoma (Broadhead et al, 2011).

1) Role, mode and mechanisms of action of physical agents inducing osteosarcoma

The role of ultraviolet and ionizing radiation in osteosarcoma is the most well-established. The connection between radiation exposure and osteosarcoma was first noted in female radium dial workers who applied radium to watch faces to make them glow. However, radiation

exposure is only thought to be responsible for a small fraction of osteosarcoma cases (2%) and is not considered a significant factor in the development of the disease in children, since an interval of 10-20 years between exposure and the development of osteosarcoma has been observed. Additionally, when radiotherapy is used to treat solid tumors in children, approximately 5.4% develop a second neoplasm, with 25% of these being sarcoma.

Both ultraviolet and ionizing radiation can sustain cancer initiation by inducing DNA damages. Of the three types of ultraviolet radiation, UVB and UVC can interfere directly with the bonding between the nucleotides in the DNA, forming dimers with two adjacent pyrimidines. UVA (and UV B) causes indirect damage to DNA via the absorption of photons by non-DNA chromophores. This generates reactive oxygen species, such as singlet oxygen or hydrogen peroxide, that oxidize DNA bases, causing mutations. The most common mutation is G-T transversion, wherein guanine is oxidized to 8-oxo-7,8-dihydroguanine (8-oxoG), hindering its pairing with cytosine. During replication, 8-oxoG pairs with adenine. When the second strand is synthesized, 8-oxoG is replaced with a thymine, leading to a G-T transversion.

Ionizing radiation acts as a clastogenic agent causing double-strand DNA breaks, leading to chromosome deletions and inversions.

2) Role, mode and mechanisms of action of chemical agents inducing osteosarcoma

The known chemical agents that are associated with osteosarcoma formation include methylcholanthrene and chromium salts (Rani et al, 1992), nickel salts (Rani et al 1993) benzo(a)pyrene (Paniaga et al, 2019), beryllium oxide (Dutra and Largent 1950), zinc beryllium silicate (Mazabraud, 1950) asbestos, and aniline dyes (Tan et al, 2009).

a. Mode and mechanism of action of methylcholanthrene and benzo(a)pyrene

Methylcholanthrene (MCA) and benzo(a)pyrene (B(a)P) are polycyclic aromatic hydrocarbons and well-known carcinogens. Both MCA and B(a)P are exogenous ligands of the aryl hydrocarbons receptor (AhR), a transcription factor that regulates gene expression. Upon ligand binding, AhR translocates into the nucleus and forms a heterodimer with its partner protein, AhR nuclear translocator. This complex is then recruited to the xenobiotic response element (XRE), a DNA-binding element located in the promoter regions of various genes. This leads to the induction of several downstream genes, including those encoding members of the cytochrome P450 (CYP) family, such as CYP1A1, CYP1A2, and CYP1B1, which play a role in the metabolism of xenobiotics. CYP1A1 supports MCA and B(a)P metabolism, leading to the formation of electrophilic metabolites, epoxides, that can be either detoxified through conjugation with cell molecules or react with DNA, forming DNA adducts and inducing genotoxic damage. The detoxification pathways are regulated by Phase-2 enzymes. The process is limited by MCA and B(a)P concentrations, when chemical concentration overcome the cell ability to foster immune-mediated adaptation and restore cell homeostasis. The activation of AhR canonical and non-canonical pathways is related to the modulation of several genes that play a key role in osteosarcoma appearance and progression (Mascolo et al, 2018; Pillo et al, 2022)

b. Mode and mechanism of action of chromium salts

Hexavalent chromium is classified as a group 1 carcinogen, and it has multiple complex mechanisms that contribute to cancer development. These mechanisms

include increased levels of oxidative stress, chromosome breaks, and DNA-adduct formation. These factors all contribute to cellular damage caused by Cr(VI). Therefore chromium is considered a genotoxic chemical (DesMarais and Costa, 2019)

c. *Mode and mechanism of action of nickel salts*

Multiple molecular mechanisms have been identified as involved in nickel-induced carcinogenicity through experimental studies. The proposed mechanisms suggest that nickel compounds enter cells, activate the receptor CaSR, triggering intracellular Ca2+ mobilization and induction of the calcium and hypoxia-inducible factor pathways. Nickel then enters the nucleus, directly binds to DNA and reacts with H2O2 to form reactive nickel-oxygen complexes, resulting in the oxidation of thymine and cytosine residues accompanied by 8-OH-dG formation. This oxidative stress severely damages DNA and inhibits DNA repair pathways. Nickel compounds also induce indirect damage through inflammation by stimulating polymorphonuclear leukocytes to produce ROS. Additionally, nickel damages heterochromatin, thereby silencing the expression of genes located near heterochromatin, which in turn leads to an epigenetic loss of histone H4 and H3 acetylation and DNA hypermethylation. As a result of gene silencing, the cell becomes more susceptible to neoplastic transformation. Furthermore, nickel down-regulates the tumor suppressor gene p53, activates the proto-oncogene c-Myc, and induces the AP-1 transcription factor, resulting in cellular proliferation and cancer development (Cameron et al, 2011) All these mechanisms have been associated to osteosarcoma (Broadhead et al, 2011; Lindsey et al, 2017; Czarnecka et al, 2020).

d. *Mode and mechanism of action of beryllium oxide and zinc beryllium silicate*

Beryllium in various forms, including beryllium sulfate, phosphate, oxide, and beryl ore, has been found to cause cancer in mice, Guinea pigs, and hamsters, although the evidence is inconclusive. The tumors induced in rabbits are mainly osteosarcomas, which are highly invasive, metastatic, and resemble human osteosarcomas in terms of histology. Beryllium compounds did not lead to mutations in Salmonella typhimurium, but they did result in genetic damage in various rodent cell cultures . It is suggested that the genotoxic effects of beryllium compounds may occur due to the binding of ionic beryllium to nucleic acids, which can lead to the inaccuracy of DNA replication (NTP, 2021))

e. *Mode and mechanism of action of asbestos*

Asbestos is composed of fine silicate fibers that are bundled together. Inhalation of asbestos fibers can cause severe health problems, including asbestosis, lung cancer and pleural mesothelioma. Most cases of mesothelioma are caused by exposure to asbestos fibers in the environment or occupational ambient air. Moreover, few cases of extraskeletal osteosarcoma arising from the pleura have been reported in subjects with a history of asbestos exposure. This is a rare type of cancer, and the differential diagnosis includes malignant pleural mesothelioma with osseous and cartilaginous elements. There are still unanswered questions about asbestos toxicity, such as why asbestos fibers are toxic to the pleura when they enter the alveoli during respiration and how asbestos causes mesothelioma, even though human mesothelial cells are easily killed upon exposure to asbestos. Asbestos toxicity is related to fibers whose length, diameter and biopersistence promote fiber accumulation in lung, since

macrophages cannot incorporate long fibers. It is now believed that the frustrated phagocytosis of asbestos fibers by macrophages prolongs inflammatory responses, creating a "mutagenic microenvironment" that can lead to mesothelial cell malignant transformation (Colacci and Vaccari, 2017). Epidemiological and genetic studies suggest that BRCA1-associated protein 1 mutations can suppress cell death in mesothelial cells and increase genomic instability in the mutagenic microenvironment, resulting in additional mutations that are associated with mesothelioma carcinogenesis (Kuroda, 2021)..

f. Mode and mechanism of action of aniline dyes

It has been observed that aniline dyes are associated with an increased risk of bladder cancer in workers who have been exposed to these substances in the workplace. Additionally, several studies have reported a causal relationship between exposure to aniline dyes and osteosarcoma. It is possible to demonstrate the formation of ultimate metabolites, their reaction with DNA, RNA, and proteins, mutagenic activity, the formation of methaemoglobin, and other acute toxic effects with practically all of these agents. However, in only a few cases has it been possible to establish a causal relationship in humans that is sufficient to classify the agent as a human carcinogen (IARC, 2010).

3) Role, mode and mechanisms of action of biological agents inducing osteosarcoma

Previously, a viral origin had been proposed for osteosarcoma, but this was later found to be due to contamination of the polio-virus vaccine that patients had received with simian virus 40 (SV40) viral units . Studies investigating the role of SV40 in the pathogenesis of mesothelioma have suggested that the detection of SV40 in human cancers may in fact be due to laboratory contamination by plasmids containing SV40 sequences.

Final considerations on human osteosarcoma etiology

The development of OS has been attributed to several mechanisms. One is the abnormal differentiation pathway of mesenchymal cells into mature osteoblasts, a process supported by the microenvironment, regulated by several cytokines, and controlled by TP53. Any disturbance in this process can deregulate the differentiation and proliferation of mesenchymal cells, leading to a malignant phenotype.

Additionally, this type of cancer is characterized by genome disorganization, aneuploidy with chromosomal alterations, deregulation of tumor suppressor genes and the cell cycle, and an absence of DNA repair. These features suggest failure in surveillance mechanisms, DNA repair, or apoptosis control during osteogenesis, allowing the survival of cells that have undergone changes during differentiation. Consequently, oncogenes and tumor suppressor genes are expressed in an abnormal manner due to genetic and epigenetic events, which leads to the deregulation of important cell signaling pathways. This creates an environment that is conducive to malignant transformation. The bone turnover rate is higher during growth, which increases the likelihood of defects in the differentiation process and signaling pathways.

A characteristic feature of OS is its histological diversity, with a mixture of different patterns in the same tumor and varying cell morphology, from normal-appearing fibroblasts to bizarre atypical cells.

However, the most significant histological feature of OS is its ability to form bone or osteoid. Moreover, osteosarcoma is heterogeneous both within the tumor and between individuals.

All environmental factors that have been claimed to play a role in osteosarcoma etiology are well recognized carcinogens able of inducing direct or indirect damages to DNA and acting as genotoxic chemicals. All of them have been classified as carcinogens by international regulatory agencies and are related to various types of tumors, including osteosarcoma, which can occur after inhalation exposure, while the oral exposure route is still a subject of debate. The most recent studies show the ability of these compounds to modulate genes and activate genetic pathways that are consistent with key molecular events in the onset and progression of osteosarcoma

Future perspectives

Further studies should be performed prior to the final evaluation of carcinogenicity.

Cell Transformation Assay (CTA) is an in vitro approach that can highlight putative genotoxic and non-genotoxic carcinogens, based on their ability to induce malignant cell oncotransformation. CTA, supported by mechanistic information, has been included in the Integrated Approach to Testing and Assessment (IATA) for non-genotoxic carcinogens (Jacobs et al, 2016, Jacobs et al, 2020, Colacci et al, 2023).

CTA measures the morphological transformation of cells, as transformed colonies or foci derived from a single cell. It is supposed to involve a multistage process that closely models some stages of in vivo carcinogenesis.

Several models have been developed and implemented since the early ‘60s. Each model offers advantages and disadvantages (Colacci et al, 2023).

All CTA models provide an easily detectable endpoint of oncotransformation, which can be used to anchor the exposure to the acquisition of the malignant phenotype

BALB/c 3T3 CTA represents a good model to highlight chemical-induced perturbations in the microenvironment and is a critical step in oncotransformation.

It could be a suitable and reliable model for highlighting the molecular and cellular key events sustaining the development of human osteosarcoma, and linking the carcinogenic activity to a phenotypic anchoring of malignancy

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