# Radiation- and Depleted Uranium-Induced Carcinogenesis Studies: Characterization of the Carcinogenic Process and Development of Medical Countermeasures

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## *ABSTRACT*

*External or internal contamination from radioactive elements during military operations or a terrorist attack is a serious threat to military and civilian populations. External radiation exposure could result from conventional military scenarios including nuclear weapons use and low-dose exposures during radiation accidents or terrorist attacks. Alternatively, internal radiation exposure could result from depleted uranium exposure via DU shrapnel wounds or inhalation. The long-term health effects of these types of radiation exposures are not well known. Furthermore, development of pharmacological countermeasures to low-dose external and internal radiological contamination is essential to the health and safety of both military and civilian populations. The purpose of these studies is to evaluate low-dose radiation or DU-induced carcinogenesis using in vitro and in vivo models, and to test safe and efficacious medical countermeasures. A third goal of these studies is to identify biomarkers of both exposure and disease development.*

*Initially, we used a human cell model (human osteoblast cells, HOS) to evaluate the carcinogenic potential of DU in vitro by assessing morphological transformation, genotoxicity (chromosomal aberrations), mutagenic (HPRT loci), and genomic instability. As a comparison, low-dose cobalt radiation, broad-beam alpha particles, and other military-projectile metals, i.e., tungsten mixtures, are being examined. Published data from our laboratory demonstrated that DU exposure in vitro to immortalized HOS cells is neoplastically transforming, mutagenic, genotoxic, and induces genomic instability. In vitro data demonstrate that radiation-specific damage is involved in the DU carcinogenic process in vitro. Furthermore, evaluation of the mutational spectrum of DU-induced HPRT mutations suggests a differential response between DU- and $^{60}$Co-irradiated cells.*

*To better assess the risk from low-dose radiation or DU, we have developed an in vivo leukemogenesis model using murine hematopoietic cells (FDC-P1). Intravenous injection of FDC-P1 cells into low-dose irradiated ($^{60}$Co 200 cGy, whole body) syngeneic DBA/2 mice was followed by the development of leukemias in 90% of all irradiated mice within 120 days. This approach was used to determine whether internal exposure to embedded DU pellets could induce leukemia in mice. For the first time, we have demonstrated leukemic transformation of hemopoietic cells in mice implanted with DU pellets (75% induction rate within 140 days).*

*Establishment of these leukemogenesis models allows us to test potentially effective medical countermeasures*

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*to radiation or DU carcinogenesis in vivo. Several agents were tested using the neoplastic transformation model in vitro and the most promising candidate (low toxicity, high effectiveness) was phenylacetate (PA). PA, a phenyl fatty acid, is a differentiation inducer that affects cellular signalling pathways. Published studies showed that PA can suppress DU transformation while clinical trials have demonstrated its safety and efficacy as an anti-tumor agent. Using the leukemogenesis models, we tested the effect of PA on leukemia latency and induction following DU or radiation exposure. Preliminary data indicate that PA at non-toxic doses can effectively reduce DU and cobalt radiation-induced leukemia (DU: DU alone 75%, DU + PA 20%; Cobalt Rad (200 cGy): Rad alone 90%, Rad + PA 10%). Further studies are necessary to confirm the results. Ongoing analysis of serum samples potentially will identify reliable biomarkers of leukemia development.*

# 1.0 INTRODUCTION

External or internal contamination from radioactive elements during military operations or a terrorist attack is a serious threat to military and civilian populations. External radiation exposure could result from conventional military scenarios including nuclear weapons use and low-dose exposures during radiation accidents or terrorist attacks. Alternatively, internal radiation exposure could result from depleted uranium exposure via DU shrapnel wounds or inhalation. The long-term health effects of these types of radiation exposures are not well known. Furthermore, development of pharmacological countermeasures to low-dose external and internal radiological contamination is essential to the health and safety of both military and civilian populations. Exposure to the types of ionizing radiation encountered during military operations may cause a number of health-related problems, but at low doses or chronic radiation exposures, the primary concern is related to the increased risk of cancer induction in soldiers. In order to develop effective medical countermeasures to low-dose radiation from external or internal contamination that is particularly relevant to military personnel, a better understanding of these types of exposures is necessary. This paper will focus on what we know about the biological and carcinogenic effects of the low-dose alpha particle emitter DU, and discuss what possible medical countermeasures are available.

# 2.0 DEPLETED URANIUM: AN INTERNAL EMITTER

## 2.1 Introduction

The radioactive heavy metal depleted uranium (DU) is used as kinetic energy penetrators in military and industrial applications [Andrew 1991]. While the use of DU in these applications has been limited to only a few countries, it has been used in recent military conflicts. Friendly-fire accidents that occurred during the 1991 Gulf War, which resulted in US soldiers with retained DU-fragments, have focused attention on the potential health effects of internalized heavy metals like DU used in military applications. Because of worldwide availability of these munitions, the United States will have to deal with an increased number of casualties from the use of these weapons. Furthermore, aerosolization of DU has lead to unsubstantiated concerns regarding environmental exposure. Since DU munitions are relatively recent additions to the list of militarily relevant metals, little is known about the health effects of this metal after internalization as embedded shrapnel.

## 2.2 DU Physical Characteristics and Exposure Hazards

DU is used by the military to fabricate armor and kinetic energy penetrators because of its effectiveness as a penetrator, a consequence of its very high density and its pyrophoric character, which results in its ignition under conditions of extreme temperature and pressure (such as that which occur upon impact with armored

targets) [Andrew 1991].

DU is a byproduct of the isotope-enrichment process that produces high specific activity uranium required by the nuclear fuels and weapons industries [NCRP 1990]. The process "depletes" DU of those isotopes, $^{235}U$ and $^{234}U$, and results in a product with a specific activity significantly less than natural uranium (0.4 μCi/g versus 0.7 μCi/g, respectively). Chemically similar to natural uranium, DU is a low specific activity radioactive heavy metal, with a density approximately 1.7-times that of lead (19 g/cm$^3$ versus 11.35 g/cm$^3$) [NCRP 1990]. However, shrapnel injury with DU would still result in an individual who is carrying an internal alpha particle emitter.

While DU's radioactivity is reduced in comparison to natural or enriched uranium, it is not eliminated. Like natural uranium, DU emits alpha, beta, and weak gamma radiation [NCRP 1990]. DU presents a minimal external radiation hazard, because the alpha particles emitted cannot penetrate the dead layer of skin (approx. 20 microns), the beta radiation is hazardous only if extended contact occurs, and the amount of the more penetrating gamma radiation is low (< 1% of total radiation). However, long-term exposure to internalized DU represents an uncertain hazard. Internalization, which can occur by ingestion, respiration, or shrapnel wounding, may be particularly injurious because of the chemical toxicity of uranium, its radiological toxicity, or a combination of the two.

## 2.3 DU Biological Effects *In Vitro*

The use of literature reviews of other internal emitters to answer questions about the health effects of DU is not appropriate, and there have been no comparison studies that would allow a realistic assessment of the risk associated with long-term exposure to embedded DU. It has been suggested that "Thorotrast" (colloidal thorium dioxide, $ThO_2$) exposures might be a useful model for embedded DU exposures. Thorotrast was a widely used radiographic agent (1900-1960), primarily emitting alpha particle radiation (90%), whose use has been linked to excess liver cancers and cancers of the reticuloendothelial and hematological systems [Andersson 1991, van Kaick 1993]. However, significant chemical differences between Thorotrast and DU preclude any simple comparison of biological effects of the two agents.

Therefore, to answer the questions regarding DU health effects, we have conducted a series of *in vitro* and *in vivo* studies to evaluate the effects of long-term DU exposure [McClain, 2002; Miller, 1996, 1998a, 1998b, 1999, 2002a, 2002b, 2001, 2002, 2003, 2004; Pellmar 1998, 1999]. Using several cellular assays including a neoplastic transformation assay, the hypoxanthine reductase transferase (HPRT) assay, and chromosomal aberration assays, i.e., micronuclei, we have examined the carcinogenic, mutagenic, and genotoxic potential of DU *in vitro* [Miller 1998a, Miller 2001, Miller 2004]. Furthermore, we have also characterized the ability of DU to induce genetic instability [Miller 2003]; this work complements results on DU's transforming potential since genetic instability is involved in development of the malignant phenotype. Figure 1 illustrates the data obtained from these studies; results with DU were compared to nickel and alpha particle exposures. These *in vitro* investigations have not only demonstrated the transforming ability [Miller 1998a] and the mutagenicity [Miller 1998b] of DU, but also its genotoxicity [Miller 2001]. Figure 1 shows that human osteoblast cells (HOS) exposed to DU-dioxide (20 μg/ml) exhibited a 17.5 ± 2.2-fold increase in transformation frequency above background transformation levels. This same exposure caused a 5.8 ± 0.72-fold increase in micronuclei formation, and a 7.3 ± 1.82-fold increase in HPRT loci in human cells. To evaluate genetic instability following DU exposure, micronuclei yields were also measured in clonal progeny of surviving DU-exposed cells. Data demonstrated that there was *de novo* genomic instability in the surviving progeny exhibiting a 5.1 ± 0.85-fold increase above control progeny. Of critical importance to an evaluation of potential DU health hazards, is our study demonstrating that alpha particle radiation is responsible for at least some of the cellular

damage induced by DU [Miller 2002]. In addition, DU induces direct damage to the genetic material manifested as increased DNA breakage or chromosomal aberrations (i.e., micronuclei) [Miller 1998a, Miller 2001]. These *in vitro* results continue to suggest that DU has carcinogenic potential.

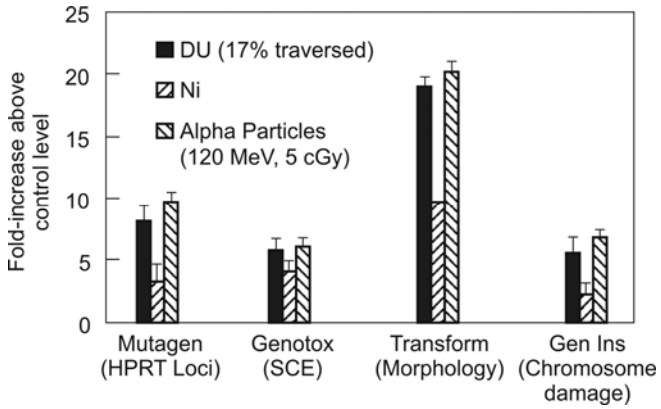


**Figure** 1. A comparison of the mutagenicity, genotoxicity, neoplastic transformation, and genomic instability resulting from cellular exposure to DU, NI, or, alpha particles is shown. Results are from greater than three experiments per endpoint and greater than three replicates were used per experiment. Cell culture (human osteoblast cells HOS), neoplastic transformation, genotoxicity, mutagenicity, and genomic instability methods have all been previously described [Miller 1998a, 1999, 20002a, 2002b, 2001, 2002, 2003, 2004]. DU dioxide 925 μg/ml; 24 hrs) and crystalline nickel (25 μg/ml, 24 hrs) were used. The microdosimetry demonstrated that during DU exposure, 17% of the cell nuclei were traversed by an alpha particle (21 μGy/nuclei). During alpha particle beam exposure 100 % of cells were traversed (24 μGy/nuclei). Significance was determined by ANOVA; significance set at $P < 0.05$. For all endpoints there was a significant increase above background; there was no significant difference between alpha particle exposure and DU.

## 2.4 DU Effects *In Vivo*

The first DU *in vivo* studies were conducted at AFRRI by Pellmar et al., [Pellmar 1999a, and Pellmar 1999b]. A rodent model of implanted DU pellets was used to assess DU toxicity. Published studies indicated that there is a rapid redistribution of DU from sites of metal pellet implantation to peripheral tissues, including the urine, kidney, bone (skull and tibia), muscle, and testicles [Pellmar 1999a, Pellmar 1999b]. DU distribution and mutagenicity studies in rats have also demonstrated a clear correlation between urine uranium content and enhanced urine mutagenicity [Miller 1998b].

Rodent mutagenicity studies which demonstrated that exposure to internalized DU was mutagenic, provide further evidence that DU has a carcinogenic potential [Miller 1998b]. To better assess this risk, we have developed an *in vivo* leukemogenesis model using murine hematopoietic cells (FDC-P1) that are dependent on stimulation by granulocyte-macrophage colony stimulating factor. Although immortalized, these cells are not tumorigenic on subcutaneous inoculation. These recent *in vivo* studies have demonstrated that DU is leukemogenic in mice causing the development of myeloid leukemia in rodents implanted with DU pellets (Table 1). Intravenous injection of FDC-P1 cells into DBA/2 mice was followed by the development of leukemias in 76% of all mice implanted with DU pellets (high dose). In contrast, only 16% of control mice developed leu-

kemia; animals implanted with a low dose of DU did not show a significant increase in leukemia development. This was the first report describing the consistent development of leukemic transformation of FDC-P1 cells when injected intravenously into DU implanted male mice. Karyotypic analysis confirmed that the leukemic cells had originated from the injected cells. Ongoing studies on the growth properties of the leukemic cells *in vitro* and *in vivo* are underway to confirm that the FDC-P1 cells had specifically undergone malignant transformation. We additionally speculate that the DU-altered host environment played a significant role in inducing leukemic transformation. Studies are being conducted to determine if alterations in the immune system are associated with exposure to the DU and if these alterations are involved in the development of leukemia. The extent of the role that alpha radiation plays in the leukemogenesis induced by DU is still, however, not fully known nor is the impact of these findings on the development of leukemia in DU-exposed humans.

**Table 1: Incidence of Leukemia in DBA/2 Mice Implanted with Depleted Uranium and Injected with FDCP1 Cells**

| Exposure[a] | No. Of Animals Implanted | No. Of Leukemic Animals[b] | Latency (Days)[c] |
|---|---|---|---|
| Control | 25 | 4 (16%) | 91-195 (143) |
| DU (High 6 pellets) | 25 | 19 (76%)* | 31-118 (75) |
| DU (Low 4 pellets) | 25 | 5 (20%) | 91-195 (143) |

[a] Animals (DBA male mice) were implanted with six (6) or four (4) pellets of DU in the hind limb; at 60 days post-pellet implantation, animals were injected with IL3 dependent hematopoietic progenitor cells (FDC-P1) cells in the tail vein. Animals were implanted with 3 DU pellets per hind limb or 2 DU pellets plus 1 tantalum pellet per hind limb. Control animals (+ FDC-P1 cells) were implanted with tantalum (6 pellets, 3 per hind limb). Number in parentheses indicates percentage of animals with leukemia upon necropsy.

[b] Evidence of leukemia by necropsy, confirmed by histology. All animals were necropsied. * Indicates significant difference

[c] Interval between FDC-P1 cell injection and death from leukemia. Number in parentheses is the median number of days at which leukemia was identified.

## 2.5 Summary

The model system studies that have been conducted in our laboratory indicate that DU has carcinogenic and leukemogenic potential, although the implications for humans wounded with DU are still unknown. These model system studies are continuing and are being extended to investigate transgenerational effects. The next phase in DU research needs to include studies that consider chemoprevention of DU-induced adverse effects. Regardless of whether DU causes the observed cellular and biological damage through chemical or radiological toxicity, the development of medical countermeasures to an internal alpha emitter like DU is critical to military deployment health.

# 3.0 DEVELOPMENT OF MEDICAL COUNTERMEASURES TO RADIATION- OR DU-INDUCED CANCER

## 3.1 Introduction

Regardless of the mode of radiation exposure to military personnel—nuclear weapons, dirty bombs, or DU—radiation-induced cancer remains a significant concern. The identification, testing, and development of cancer chemopreventive agents are crucial to military force health protection. Advances in our understanding of the molecular biology of cancer have provided new approaches to cancer prevention by the identification of molecular biomarkers or abnormalities that are linked to the carcinogenic process. The identification and characterization of these aberrant changes provide novel targets for chemopreventive drugs.

One of these aberrant changes is altered cellular signal transduction. Aberrant signal transduction has been linked to tumorigenesis [Adjei 2001, Alonso 1998, Vanden Heuvel 1999]. These abnormalities in signal transduction may involve a) inappropriate expression and activation of receptors, i.e., tyrosine kinase receptor,

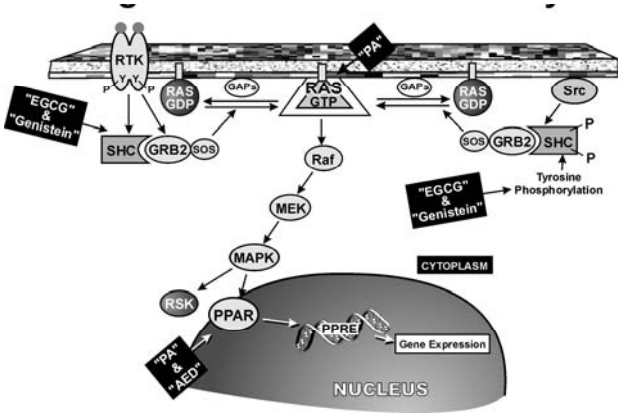


**Figure 2: Signal Transduction Pathway**

peroxisome proliferator-activated receptor; b) oncogene activation, i.e., *ras*, src; and/or c) point-mutated transducers such as G proteins [Adjei 2001, Alonso 1998, Vanden Heuvel 1999]. Each of these activities is an important subset of the common signal transduction pathways that have been linked to cancer biology. The common theme is the coupling of the abnormal signal with the transforming event(s). The Ras pathway is of particular interest because it is a key signalling pathway in carcinogenesis. Furthermore, this pathway is not a simple linear one but involves complex signalling circuitry including tyrosine kinase, PPARs, and mitogen-activated protein kinases (MAPK) [Vanden Heuvel 1999] (Figure 2). Individually, these pathways have each been linked to carcinogenesis [Adjei 2001, Alonso 1998, Vanden Heuvel 1999]. Therefore, the linkage between the aberrant signalling pathway and malignant transformation presents an attractive target for anticancer drug development.

## 3.2 Candidate Agents: Phenylacetate and Epigallocatechin Gallate

Phenylacetate (PA) is an attractive candidate for our studies because it has low clinical toxicity, has exhibited high antitumor activity in cellular, animal, and clinical studies [Samid 1997, Samid 1999, Thibault 1995, Thi-

bault 1999]. PA, a phenyl fatty acid, is a differentiation inducer that affects cellular signalling pathways. Published studies from our laboratory showed that PA can suppress DU neoplastic cell transformation while clinical trials have demonstrated its safety and efficacy as an anti-tumor agent [Miller 2001]. Additionally, we have published PA's effects on the *ras* signalling pathway (p21ras protein levels) and the peroxisome proliferator receptor [Miller 2001, Shack 1995].

The polyphenol, epigallocathechin-3 gallate (EGCG), a major constituent of green tea, is also an attractive candidate for low-dose radiation chemoprevention. EGCG exhibits chemopreventive effects in chemically-induced cell transformation and animal tumorigenesis studies [Lin 2000, Moyers 2004, Sakurai 2005]. EGCG treatment affects the cell-signalling pathway by affecting both *ras* gene expression and protein tyrosine kinase levels [Lin 2000, Moyers 2004]. Both of these agents are excellent candidates as potential radioprotectors of the late effects of radiation exposure.

## 3.3 Suppression of Radiation- or DU-Induced Neoplastic Transformation

PA has been previously shown by others to suppress chemically-induced carcinogenesis both in culture and in mice [Prasanna 1995]. The ability of PA to suppress DU-induced neoplastic transformation has also been demonstrated by our laboratory. Data showed that incubation of human cells (HOS) with PA reduced DU-induced transformation (Table 2).

**Table 2: Modulating Effects of Phenylacetate on Depleted Uranium-Induced Neoplastic Transformation in Human Cells[a]**

| Exposure of DU (25 μM, 24 hr) | Exposure Time of PA (2.5 mM) | Surviving Fraction | Transformation Frequency per Surviving Cell X $10^{-4}$ |
|---|---|---|---|
| None | 0 hr | 0.99 (± 0.04) | 4.17 (± 0.43) |
| DU-dioxide | 0 hr | 0.89 (± 0.05) | 77.3 (± 5.5)* |
| DU-dioxide | 24 hr | 0.91 (± 0.06) | 52.5 (± 4.9)* |
| DU-dioxide | 6 weeks | 0.95 (± 0.04) | 5.15 (± 0.55) |

[a] Human osteoblast cells (HOS; immortalized, nontumorigenic) were exposed to a transforming dose of DU (25 μM, 24 hr) and then were rinsed with PBS; the cultures were re-incubated for 0, 24 hr, or 6 weeks with PA 2.5 mM until neoplastic transformation could be assessed. Normal time to develop transformed colonies is 6 weeks. Control cells (no DU, no PA) were used to determine spontaneous transformation frequency (background). Significance was determined by ANOVA; significance set at $P < 0.05$. * Indicates a significant difference.

Cellular treatment with PA reduced DU-induced transformation to spontaneous levels (77. 3 x $10^{-4}$ and 5.15 x $10^{-4}$, respectively) when PA was present during the entire 6-week incubation period. When PA was present only during the 24-hr period of DU exposure, there was a moderate reduction in transformation frequency in comparison to spontaneous transformation (52.5 x $10^{-4}$ ± 4.9 and 4.17 x $10^{-4}$ ± 0.43, respectively). These results demonstrated the substantial suppressive effect of PA on DU-induced transformation using a cell model.

To extend the results obtained with PA in a DU-induced transformation assay to a radiation-induced transformation assay, we examined whether PA could suppress alpha particle- or x ray-induced neoplastic transforma-

tion. This would allow us to ascertain whether PA would exhibit effectiveness as a suppressor of radiation-induced transformation and, secondly, whether PA's effectiveness was similar to both a high (alpha particles) and low (x rays) LET radiation dose. Data are shown in Figure 3. Cellular exposure to alpha particles (5 cGy, 120 MeV) or x rays (50 cGy, 250 kVp) induced neoplastic transformation in HOS cells and resulted in a transformation frequency of 99.2 ± 8.9 and 16.4 ± 1.9, respectively. Incubation of HOS cells with PA after radiation exposure was effective in reducing both alpha particle- and x ray-induced transformation; treatment with PA significantly reduced alpha particle-induced transformation (2 Gy, 120 MeV) although not to spontaneous levels ($40.2 \times 10^{-4}$ and $5.15 \times 10^{-4}$, respectively). In contrast, PA treatment was able to reduce x ray-induced transformation frequency to spontaneous levels ($16.4 \times 10^{-4}$ and $5.15 \times 10^{-4}$, respectively). These results indicate that PA treatment can effectively suppress radiation-induced neoplastic transformation. The observed differences between the alpha particle and x ray-suppressive response of PA also suggest that PA may be less effective against a high LET radiation like alpha particles. Further studies including an assessment of PA dose and timing effects on radiation transformation are necessary to answer these questions.

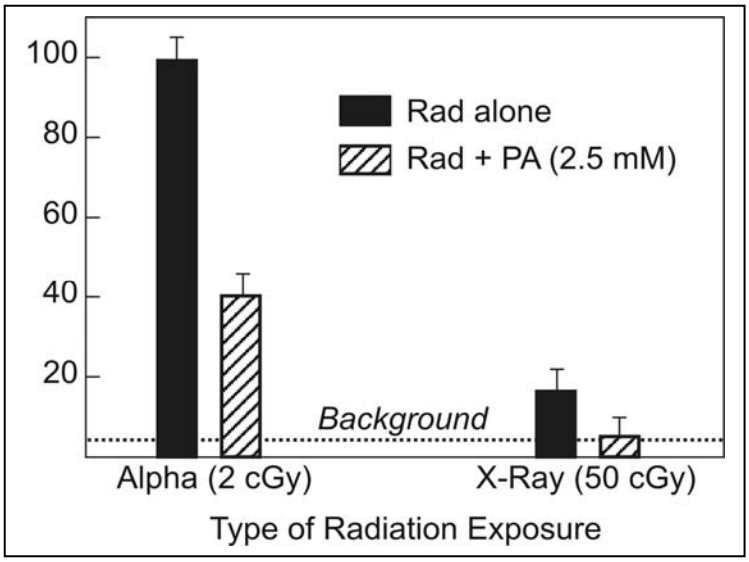


**Figure 3.** Modulation of Neoplastic cell transformation by PA (2.5 mM 6 weeks) after exposure to either alpha particles (2 cGy, 120 MeV broad beam) or X-rays (50 cGy 150 MeV) is demonstrated in this figure. HOS cells were irradiated to induce transformation. Cell culture, neoplastic transformation, and alpha radiation (Columbia University, Radiological Research Accelerator Facility (RARAF)) have been previously described [Miller 1998a, 1999, 2001, 2004]. Three experiments with three replicates per point were done. Significance was determined by ANOVA; significance set at $P < 0.05$. There was a significant difference between alpha-induced and X-ray induced transformation and control; PA treatment caused a significant reduction in transformation.

Our laboratory currently is evaluating the ability of EGCG, the green tea extract, to inhibit radiation-induced neoplastic transformation. Initial studies have demonstrated that EGCG is an effective suppressor of $^{60}$Cobalt-induced neoplastic transformation (data not shown). The ongoing investigations with both PA and EGCG will provide valuable information that will allow us to develop and test effective medical countermeasures to ra-

diation-induced neoplastic disease.

### 3.4 Summary

Development of medical strategies that prevent or decrease radiation-induced late health effects is critical to the US military. Radiation exposure is an ongoing threat to military personnel in an occupational (nuclear reactors, depleted uranium) or warfare (nuclear weapons) scenario. Our work developing and testing non-toxic medical countermeasures like PA and EGCG to radiation-induced cancer will help to identify potential prophylaxis that would provide assistance to the medical and operational military community.

## 4.0 CONCLUSIONS

Our laboratory currently is assessing the extent of the health hazards of low-dose radiation like DU that could result from exposure to radiation during military operations. External radiation exposure could result from conventional military scenarios including nuclear weapons use and low-dose exposures during radiation accidents or terrorist attacks. Alternatively, internal radiation exposure could result from depleted uranium exposure via DU shrapnel wounds or inhalation. The long-term health effects of these types of radiation exposures are not well known. Using cellular and animal models, we are investigating these exposures. Furthermore, development of pharmacological countermeasures to low-dose external and internal radiological contamination is essential to the health and safety of both military and civilian populations. Studies from our laboratory have identified two promising non-toxic agents that may be effective in preventing radiation-induced cancer. Further studies are warranted and will provide information regarding toxicity, efficacy, and application.

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