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Research Article

The Herbicide Picloram: Growth Inhibition, Cytotoxicity and Apoptosis Activity of Picloram in Normal and Virus-Transformed Cells in Tissue Culture: Possible Carcinogenicity and Mutagenicity for Eukarya

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Abstract

The data presented here indicate that picloram is cytotoxic for cultured mammalian cells, as it inhibits ribosomal protein synthesis, in particular MPS1/RS27, and other proteins of the 'small ribosomal' subunit; it also induces pronounced toxicity of the nucleoli, and apoptosis is evident in this model system. The model consists of NRK cells and cells transformed by DNA and RNA viruses (SV-NRK and K-NRK), respectively. Analogues of picloram were also cytotoxic. NRK cells when exposed to picloram show arrest of cell growth. SV-NRK and K-NRK cells show a faster and much more pronounced cytotoxicity than NRK cells. The genotoxic effects of picloram cannot be counteracted by picolinic acid, an anti-cancer and anti-viral agent derived from enzymatic reactions of tryptophan in vivo. Analogues of picloram have more severe cytotoxicity in both normal and virally transformed cells. They also induced growth arrest and apoptosis in both SV-NRK and K-NRK. Apoptosis in these cell lines clearly indicates genotoxicity that may result in carcinogenesis. It appears that prior testing of picloram, for carcinogenesis, which was done in animals, is a method incaple of detecting carcinogenicity. The author proposes new technologies such as specific ribosomal protein synthesis, genome context analysis, and determination of the structure of the nucleoli, the place of synthesis of ribosomal subunits. These methods may be useful to prevent the introduction of genotoxic herbicides in the food chain. Finally, the results suggest that picloram induces genotoxic effects not previously recognized.

Keywords: Picloram; Nucleolus; Metallopanstimulin-1(MPS1); Picolinic acid; Apoptosis; Carcinogenesis

Abbreviations

MPS-1/S27-Ribosomal Protein; NRK-Normal Rat Kidney; SV-NRK-Simian-Virus 40-Transformed NRK; K-NRK- Kirsten Sarcoma Virus-Transformed NRK; SF - Substructure Finding; SOD-Superoxide Dismutase

Introduction

Picloram (4-amino-3,5,6-trichloropicolinic acid) is a broad-spectrum systemic herbicide, used to control a wide range of broad-leaved weeds. Picloram is a chlorinated derivative of picolinic acid, a naturally occurring derivative of tryptophan in mammalian cells. Thus, picloram belongs to the pyridine family of herbicides and can interfere with the metabolism of picolinic acid [1-5].

Studies have shown that picloram is apparently of moderate toxicity to mammalian tissues [1]. It is registered by the EPA only for non-food use to control broad-leaf weeds and woody plants [2,3]. The chemical appears to act by replacing the plant growth hormone indoleacetic acid [2]. However, the mechanism of action of picloram may be much more complex at the molecular level, as it also inhibits protein synthesis in plants and in mammalian cells [1-5]. The exact mechanism of action of inhibition of protein synthesis is unknown and the levels of evidence of carcinogenicity have not been well defined.

In this report, we document the growth arrest, inhibition of protein synthesis, cytotoxicity and apoptosis of picloram toward normal and virally-transformed mammalian cells in tissue culture. Apoptosis induced by picloram clearly shows the relationship between inhibition of protein synthesis and growth arrest. Furthermore, taken together with other recent work by Berthon et al [6] using genome context analysis to determined DNA damage, and repair, they correlate with the cytotoxic effects of picloram in Eukaryote (plants and animal cells) with DNA repair and specific ribosomal proteins such as MPS1/S27 ribosomal protein which possesses extra-ribosomal functions [7-14]. Our results in conjunction with the results of Berthon et al [6-14], who utilized genome context analysis using super-computers and 20 billion base pairs of DNA analysis, together with our studies demonstrate that picloram, most likely, is a carcinogenic agent for Eukarya (plant, animal and human) cells in intact biological systems. Thus, taken together with numerous other results, the data presented here indicate that picloram is a mutagenic and carcinogenic agent that has been introduced in the

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edible food change. Finally, the mechanisms of action of picloram is not as simple as previously thought, and such complex mechanism of action in Eukaria is potentially dangerous to mammalian cell systems, including humans.

Materials and Methods

Materials

Chemically defined media was purchased from Sigma-Aldrich St. Louis MO, USA. The medium consisted in a vitamin-and amino-acid-supplemented balanced salt solution, supplemented with transferrin (final concentration, 50 µg/mL), and insulin. Picloram was also obtained from Sigma-Aldrich.

Cells

On day one, cells were grown in DME containing 10% (v/v) calf serum. Stock cultures were started from frozen cells at approximately 2-months intervals. NRK-B cells clone 2 were purchased from Biotech Research Inc., Rockville, MD, USA. SV-NRK P8 C12T17 (SV-NRK) and K-NRK Cl32 (K-NRK), were purchased from the American Type Culture Collection, Rockville, MD. Cells were counted with a Coulter counter. The cells were plated at 100,000 cells per 60-mm Petri dish for all cell lines. Each cell line was plated in forty, 60-mm dish at the indicated density, and counted in duplicate with a Coulter counter. The cells attached to the Petri dishes were counted, after trypsinization, and in parallel dishes were also used for apoptosis determinations, following specific protocols, as described below; 40 dishes per apotosis experiment were measured in duplicate. The supernatants, containing floating cells, were saved and used for apoptosis determinations, as detailed below.

Apoptosis

The CytoSpin Kit, for apoptosis determination was purchased from Sigma-Aldrich, St. Louis, MO, USA. The Apotosis-CytoSpin was performed following the manufacturer's specifications.

Results

Effects of picloram on growth of Normal and virally-transformed cells

NRK cells, and its virally transformed derivatives (Simian virus-40; SV40-NRK, and Kirsten sarcoma virus, K-NRK) cultures, were incubated with picloram, one day after plating, previous media changed and 2 washes with defined media. The cells were exposed to defined media, with and without picloram, from time, t = 0 to 72 h. The concentrations of picloram were from 0.5, 1.0 to 3 mM. Before counting, the media was removed and the supernatants were collected after centrifugation for apoptosis studies. Cells attached to the Petri dishes were counted at 0, 24, 48 and 72 h after addition of picloram; control, untreated cells were also counted at the same time points. The growth arrest induced by 3.0 mM picloram was accompanied by notable cytotoxic morphological changes, such as changes in cell shape (flattening, elongation, and prominent nucleoli) and increased in the number of intracellular vacuoles, which indicated cytotoxicity by 3 mM picloram.

NRK cells (control, normal), showed < 20% growth inhibition after treatment with 0.5 mM or 1 mM picloram for 48 h. However, 3 mM picloram strongly inhibited NRK cell growth by 48 h (50%), and 80% at 72 h. The NRK cells showed signs of cytotocixity in the presence of 3 mM picloram, but were not as pronounced as the SV-NRK or K-RNK cells.

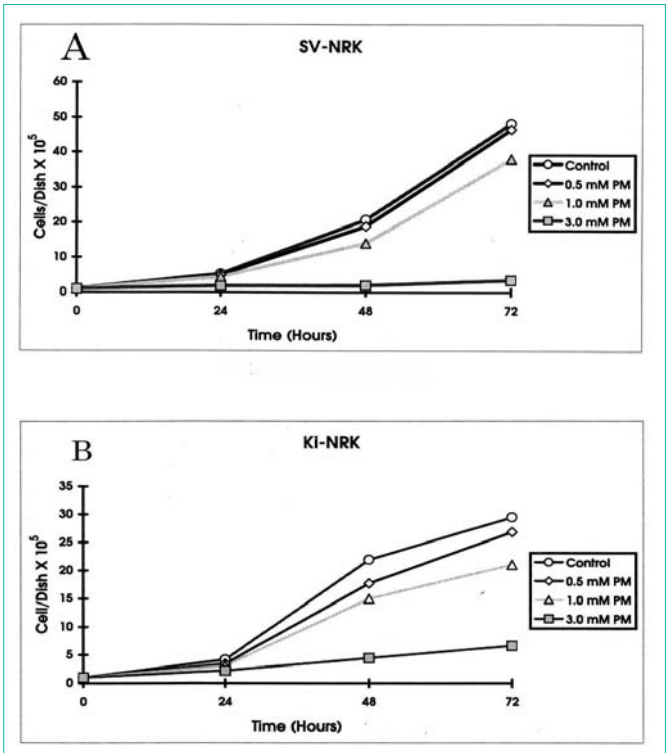


Figure 1: Dose- and time-dependence of growth inhibition in SV-NRK (A), and K-NRK (B) cells treated with picloram. Cells were plated at 7.5 x 10^4 cells/60 mm dish; 24 h later, the medium was removed, and new media containing picloram at the indicated concentrations were added. The control cultures were treated identically but without the herbicide. Cell counts were determined at the indicated times; each point is the average of duplicate measurements from 2 cultures. Control; 0.1 mM picloram; 0.5 mM picloram, 3 mM picloram. As indicated in the insets, the symbols indicate the controls, and each of the doses of picloram used in the experiments.

Figure 1A shows the time- and dose-dependent effects of exposure of SV-NRK cells to 0.5, 1 and 3 mM picloram. 3 mM picloram completely arrested cell growth at 24, 48 and 72 h. The growth arrest by 3 mM picloram of the treated cells, was greater than 90% in SV-NRK cells, in comparison to control untreated SV-NRK cells. The majority of SV-NRK cells showed signs of cytotoxicity with 3mM picloram, and was floating and/or disintegrated in the supernatants. Morphological changes of SV-NRK were conspicuous and the cells showed flattening when attached to the Petri dishes, also showing long extensions attached to the Petri dish.

Figure 1B shows the time- and dose-dependent effects of exposure to picloram on K-NRK cells from 0.5, 1, to 3 mM picloram. Picloram at 0.5 mM had a significant inhibitory effect on growth of K-NRK cells with respect to control untreated K-NRK cells, from 48 to 72 h. Picloram at 1 mM showed even a more substantial reduction in K-NRK cell number from 48 to 72 h. Exposure of K-NRK cells to 3 mM picloram arrested cell growth at 24, 48 and 72 h by >90%. Exposure of K-NRK cells to 3 mM picloram, showed intense signs of cytotoxicity, and the majority of the cells floated in the supernatant and/or disintegrated by 48 to 72 h.

In all conditions using 3 mM picloram, there were signs in

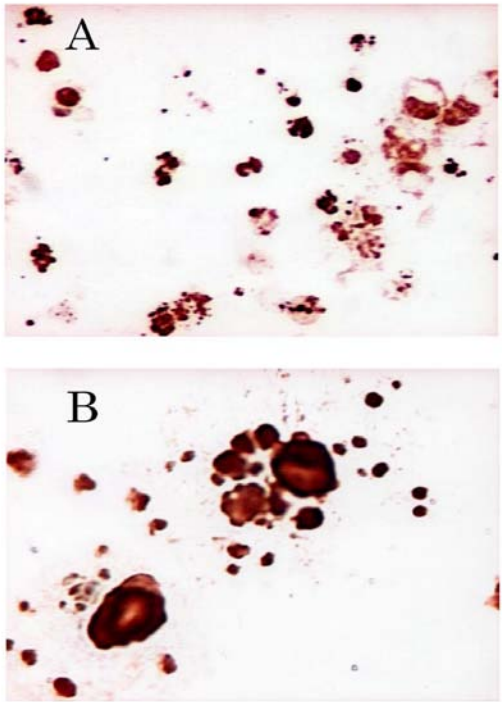


Figure 2: Induction of apoptosis by picloram in Kirsten sarcoma virus transformed NRK cells (K-NRK), growing in monolayer cultures. The cells were exposed to 3 mM picloram for 24 h. One of the characteristics of apoptotic cells is fragmentation of DNA. This fragmentation of DNA produces free 3'-OH groups. These groups were end-labeled by the Klenow polymerase using biotinylated dNTPs. The incorporated biotin was reacted with a peroxidase-streptavidin conjugate, and staining was produced by a DAB H_2O_2 substrate. K-NRK cells showed apoptosis within 24 hr of exposure to 3 mM picloram. Pictures show floating apoptotic cells' nuclei (A) Mag. x 200; (B) Mag. X 400. For details see the text.

virtually every treated SV-NRK or K-NRK of nucleoli cytotoxicity, as shown by the observation of dark, condensed nucleoli in greater than 80% of these cells (data not shown), indicating disruption in ribosomal protein synthesis.

Apoptosis

When SV-NRK and K-NRK cells were treated with 3 mM picloram, apoptosis was observed, and was more prominently in the case of 3 mM picloram.

Figure 2A and Figure 2B show the effects of 3 mM picloram on K-NRK cells. The nuclear changes observed are due to activation of a Ca 2+/Mg 2+/H + dependent-endonuclease which produces double strand breaks in the internucleosomal linker DNA, creating oligonucleotides with lengths in multiples of approximately 200 bp. Thus, apoptotic cells show a "DNA ladder" corresponding to DNA fragments of multiples of 200 bp [200 x 2^n].

Figure 2A and Figure 2B show induction of apoptosis by 3 mM picloram in virally transformed K-NRK cells at 200x and 400x Mag., respectively, on floating cells of the supernats of Petri dishes, after biochemical processing (CytoSpin procedure). One of the characteristics of apoptotic cells is fragmentation of DNA. This fragmentation of DNA produces free, 3'-OH groups. These groups were labeled by the Klenow polymerase using biotinylated dNTPs. The incorporated biotin was reacted with a peroxidase-streptavidin conjugate, and staining was produced by a DAB H2O2 substrate. K-NRK showed apoptosis within 24 h of exposure to 3 mM picloram.

Picolinic acid, Picloram and analogs

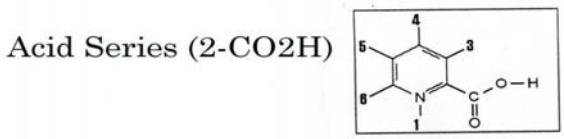
We have undertaken experiments to identify substructures of picolinic acid and picloram based on the Picolinic acid series (2-CO_2H). Table 1 show all variations of positions 1, 3, 4, 5 and 6, using Substructure Finding (SF) software (purchased from Sigma-Aldrich, St. Louis, MO, USA). The structures found after the search, are shown in Table 1, and were the basis for further investigation of analogues. The carboxylate group at position 2 is fixed. After initial search of Picolinic acid analogs, the commercially available analogs listed in Table 1 were used. Holding the 2 position inviolate as carboxylate reduced the number of pertinent candidate analogs to 48 [15,16]. These compounds are listed in Table 1, entitled 'Acid Series (2-CO_2H)'. In this Table 1, the first raw shows Picolinic acid as the first compound in the left and for comparison purposes, Picloram is shown in the same first line on the right line. All the other Picolinic acid and Picloram analogs are listed in the same Table 1. In this investigation, we focused on comparing the cytotoxic, growth inhibition, and apoptotic properties of picloram and picolinic acid in tissue culture. All analogs substituted at position 1 were eliminated, as the chelating properties of picolinic acid, picloram and analogs prevent the chelation of transition metal ions, and also other essential functions of the N at position 1. From the preliminary testing of the analogs shown in Table 1, other than Picolinic acid and Picloram, the remaining 46 analogs have well defined inhibition of nucleoli protein synthesis and genotoxicity (data not shown).

Discussion

There is no scientific doubt that herbicides used to control a wide arrange of weeds, are to a certain extent not only toxic for plants and animals, but also for humans. However, their cytotoxicity has different degrees and depends upon the capacity of the cells to enzymatically neutralize and/or degrade selectively the herbicides [2]. Thus, it is clear that above certain threshold of herbicidal toxicity the harmful properties of the herbicides to produce cellular damage might be severe, and that they can accumulate and induced DNA or mRNA mutations, or inhibition of protein synthesis; all these toxic factors result in genotoxicity [1-5]. The type of chemical compound and its intrinsic high or low toxicity for the target organism to be intoxicated is a function of the dose- and time-dependent exposure to the herbicide.

Previously, and also at present time, bioassay techniques with laboratory animals were used for determining possible carcinogenesis of numerous herbicides [1-4]. This type of bioassay is done by administering the herbicide(s) or the "test compound" in the feed of various types of genetically stable rats and mice, and the animals are observed for set time periods. It is worth noting that in the case of picloram, these types of bioassays have shown an increased incidence of hepatic neoplastic nodules which were observed in picloram-treated male and female rats as compared to untreated control mice and rats [1]. These hepatic lesions were considered to be benign tumors [1], but with current, more powerful and sophisticated techniques, to determine DNA mutagenesis, the interpretation of these results have severe limitations. For example, we know for the last 30 years, that benign polyps from the colon develop into highly

Table 1: Picolinic acid and Picloram analogs.
Acid Series (2-CO_2H)



Picolinic acid

3	4	5	6
CO2H	H	H	H
H	CO2H	H	H
H	H	CO2H	H
H	H	H	CO2H
CO2H	H	CO2H	CO2H
CH3	H	H	H
H	H	H	CH3
H	Et	H	H
H	H	n-Propyl	H
H	H	n-Bu	H
H	H	Et	H
H	H	n-Heptyl	H
H	H	4-n-Hexylcyclohexyl	H
H	H	cyclohexyl	H
H	t-BuOCONHCH2	H	H
H	H	H	F
H	Cl	H	H
H	H	H	Cl
Cl	H	Cl	H

Picloram

3	4	5	6
CL	NH2	CL	CL
Cl	H	H	Cl
Cl	Cl	Cl	Cl
H	H	H	Br
I	H	H	H
OH	H	H	H
H	H	OH	H
H	H	H	OH (=O)
nPrO	H	H	H
NH2	H	H	H
H	NH2	H	H
H	H	NH2	H
H	NO2	H	H
C6H5CH2S	H	H	H
CONH2	H	CO2CH3	H
H	H	H	CO2CH3
H	H	H	CO2Et

malignant colon carcinoma by additional mutations of the "benign" polyps. Thus, these methods of testing herbicides in animals, although important, suffer serious experimental defects that prevent investigators to arrive at solid conclusions on whether the herbicides are carcinogenic, mutagenic, or not. Moreover, some hepatocellular carcinomas were found in some animals treated with picloram [1,3], but the results were statistically not significant. Nevertheless, the results with picloram showed that apparently benign tumors can developed into malignant tumors [1], similarly as the case of polyps-colon cancer. The investigators concluded that picloram is able to induce the development of benign tumors in rats, but the data was not sufficient to conclude that picloram was carcinogenic or mutagenic in these animals [1,2].

Advances in new technologies, such as Genomics and Proteomics clearly indicate that to determine unambiguously the toxicity of herbicides, as far as carcinogenicity is concern, methods have to be used to determine the minimal threshold of mutagenicity, carcinogenicity, inhibition of ribosomal protein synthesis, and nucleoli alterations, produced by herbicides in Eukaryotic genomes in both plant and animal cells. Conspicuously, there is no published method that can distinguish any difference between an herbicide uses in destruction of weeds versus a chemotherapeutic agent used to treat cancer. Thus, it is not surprising that the separation of herbicides as weed killers and chemotherapeutic cancer agents as cells killers is an artifice of applying statistically deficient methods to the structure and stability of covalent complexes [15,16], which are undeniable defined by the wave functions valence-bond structure clearly defined by quantum mechanics chemistry [15,16]. New reliable approaches will have to be developed, as they are now, to avoid deceptive concepts and erroneous statistics applications, which only lead to legal and useless arguments, regulations, and not true physic-chemical undeniable scientific methodologies [15,16] that will provide unambiguous data. As follows, a few examples will be cited of advances towards that goal: (i) Genome Context Analysis [6]; (ii)genotoxic effects in the complex factory of ribosomes, [16-23], radicated in the nucleoli [16,18,19], and (iii) non-ribosomal functions of ribosomal proteins [9], will be pointed out as advanced techniques that will add the detailed and certainty of unwanted toxicity of herbicides and chemotherapeutic agents before being used, by relying in quantum mechanics, chemical bond facts [15,16], rather than statistical artifices that contradict the basic physic-chemical actions of herbicides. Undeniable, the author believes that the nature of the chemical bond at the molecular orbital-resonance level will be useful to solve the problem of undetected toxicity of herbicides, pesticides and chemotherapeutic agents [15,16].

Genome Context Analysis proposed by Berthon et al [6]. This analysis has shown that in Archaea and Eukarya previously unrecognized links between DNA replication and ribosomal translation. Furthermore, these results indicate interactions of proteins involved in DNA replication between each other or with various proteins involved in DNA repair or transcription, such as Metallopanstimulin-1 (MPS1/S27), which is involved in repair of DNA [6,9,16]. These proteins are in highly conserved clusters of genes coding for proteins involved in translation, transcription, and DNA repair and ribosome biogenesis [6]. Moreover, they are systematically contiguous phylogenetically in Eukarya (plants, animals and humans). Berthon et al also found that the MPS1 (S27) ribosomal protein has been a component of these clusters since the appearance of Archaea life on earth, 5 billion years ago [6].

The Nucleolus: the ribosomal factory plays a critical role in herbicide and chemotherapeutic agent genotoxicity [15-22]. Within the nucleoli critical events of ribosome biogenesis take place [18]. At present, the location in the nucleoli of specific proteins are being individually counted and a correlation between the numbers of molecules in normal cells versus cancer cells is being established by special immune-microscopic techniques [18]. This detailed information is allowing the quantitative differentiation between

a normal cell, with a cell that is in the process of carcinogenesis, with a certainty previously unavailable. The same method can be used to determine genotoxic effects of herbicides, pesticides and chemotherapeutic agents for agribusiness uses and chemotherapeutic agents for treating cancer in patients, respectively. The author believes that in the years to come, these methods will be of critical importance to determine viability of use in the environment in massive scale for combating pests and plagues in crops without producing insidious long term toxicity to humans and the environment. Chemotherapy will also benefit, as using these methods for early testing of new agents will save money and prevent untoward or unexpectedly deadly consequences for patients.

Extra-Ribosomal Functions of selected ribosomal proteins: a number of specific ribosomal proteins have special extra ribosomal functions and are involved in herbicide cytotoxicity, chemotherapeutic cytotoxicity, and are critical for viral replication of pathogenic viruses. In 1983, Fernandez-Pol, et al found that MPS1/S27, is instrumental in growth regulation and carcinogenesis in animals and humans [11]. Further work lead to extra-ribosomal functions of MPS1/S27 and its absence of functions in mutated forms of this protein. MPS1/S27 and analogs have a critical role in controlling survival events in Archaea and eukaryotic animal and plant cells [6,9].

Unexpectedly, a conserved cluster of genes coding for proteins involved in translation and ribosome biogenesis (MPS1/S27, L44E, aIF- alpha, Nop10), was found almost systematically contiguous to the group of genes coding for PCNA, PriS, and Gins 15. This cluster encodes proteins conserved in Archaea and Eukarya [6]. These data lead to the proposal that MPS1/S27 and its associated ribosomal proteins may be used to determine carcinogenicity and mutagenicity of herbicides and pesticides that damage these DNA clusters [9,10], as they are involved in transcription and DNA repair [13].

The nucleoli are involved in the complex process of protein synthesis; as the ribosomes are assemble in the nucleoli. Then, it is suggested that the use of genome context analysis, in conjunction with pathological microscopic advanced techniques used now to compare damaging radiation such as UVA, and gamma-radiation, and differentiating normal cells from cancer cells with a great degree of certainty, may be equally used to determine genotoxicity of potential herbicides and pesticides which will lead to safer herbicides. In addition, their specific mechanism of action and side effects will be clearly known, preventing animal and human exposure to deleterious compounds prior to release of the herbicides and pesticides to the environment.

The experiments presented here demonstrate that the same concentrations of picloram are cytotoxicity to both SV40-NRK and K-NRK virus-transformed cells. Picloram is less toxic to non-transformed NRK cells under similar conditions. Although the selective mechanism of action of picloram in normal versus virally transformed cells is unknown, the tolerance of NRK cells to picloram may be associated with high intracellular Superoxide dismutase (SOD) activity in NRK cells [7]. The cytotoxicity of picloram to SV-NRK and K-NRK may be due to low levels of SOD in virally-transformed cells [7]. In addition to the superoxide anion, hydrogen peroxide, and hydroxyl radicals can be produced by the Fenton reaction involving pyridine carboxylic acids, such as picloram, in the presence of Fe^{2+} / Fe^{3+} [11], The radicals produced by the Fenton reaction are highly toxic [11]. The differences in reactive oxygen species among NRK, SV-NRK and K-NRK may be the cause of the differential cytotoxicity of picloram in these cell types [7]. However, other mechanism of cytotoxicity of picloram is also possible, like disruption of Zinc-Finger Proteins and interference with the naturally occurring anti-viral and anti-cancer compound Picolinic acid [11].

Conclusion

Taken together with published results [6,11], the data shown in this paper indicate that picloram inhibits protein synthesis in both plant and animal cells, which result in cytotoxicity and apoptosis of normal and virally-transformed cells. The genotoxic effects of picloram cannot be counteracted by endogenous Picolinic acid, an anti-cancer and anti-viral agent [11], and analogues of picloram have similar or more severe cytotoxicity and also induced apoptosis [Table 1]. As the majority if not all herbicides created since the 1900's and after II World War, inclusive Agent Orange used in Vietnam for deforestation, have been ban by USA Government agencies for their catastrophic consequences to life in general, it is clear that even today, there is a failure in the identification of herbicides and pesticides that are safe for animals and humans. It is clear that the safety tests used in the past had failed in their goals, and new tests should be developed to prevent long term genotoxicity to animal and humans with a degree of certainty such that long term genetic defects and carcinogenesis will not become a permanent feature of the population of this planet, with obvious deleterious consequences and degeneration of species exposed to unsuspected toxic herbicides and pesticides.

Acknowledgment

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