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# A radical approach to cancer

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## Summary

Reactive oxygen species are known to be potentially dangerous, but are also needed for signal-transduction pathways. Tumor cells have relatively low amounts of superoxide dismutase (SOD), which quenches superoxide anion ($O_2^{-\bullet}$), and as a result of a higher level of aerobic metabolism, higher concentrations of $O_2^{-\bullet}$, compared to normal cells. But this may not be true of all tumor cells. Some tumor cells have relatively higher amounts of vitamin E, a potent anti-oxidant, and a higher level of anaerobic metabolism, resulting in a balance that is tilted more towards higher anti-oxidant capacity. In both instances of higher aerobic and anaerobic metabolism methods designed to augment free radical generation in tumor cells can cause their death. It is suggested that free radicals and lipid peroxides suppress the expression of Bcl-2, activate caspases and shorten telomere, and thus inducing apoptosis of tumor cells. Ionizing radiation, anthracyclines, bleomycin and cytokines produce free radicals and thus are useful as anti-cancer agents. But they also produce many side-effects. 2-methoxyoestradiol and polyunsaturated fatty acids (PUFAs) inhibit SODs and cause an increase of $O_2^{-\bullet}$ in tumor cells leading to their death. In addition, PUFAs (especially gamma-linolenic acid), 2-methoxy-oestradiol and thalidomide may possess anti-angiogenic activity. This suggests that free radicals can suppress angiogenesis. Limited clinical studies done with gamma-linolenic acid showed that it can regress human brain gliomas without any significant side-effects. Thus, PUFAs, thalidomide and 2-methoxyoestradiol or their derivatives may offer a new radical approach to the treatment of cancer.

**key words:** **free radicals • 2-mehtoxyestradiol • polyunsaturated • fatty acids • superoxide dismutase • tumor cells**

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## BACKGROUND

During the therapy of malignant tumors it is desired that agents attack preferentially tumor cells without exerting adverse effects on normal cells. But, this is rarely achieved with the currently available drugs and radiation. Ionizing radiation and certain drugs, such as anthracyclines and bleomycin, produce free radicals [1–3] and thus, bring about their tumoricidal action. But, at the same time, these free radicals are responsible for the side-effects seen with these agents. Thus, both radiation and anthracyclines can induce the production of free radicals both in the normal and tumor cells.

Free radicals are potentially dangerous by-products of cellular metabolism that have direct effects on cell growth and development, cell survival and have a significant role in the pathogenesis of atherosclerosis, cancer, aging and several other conditions, including inflammatory diseases [4–6]. Free radicals are generated by aerobic organisms during the production of ATP (adenosine triphosphate) in mitochondria. During the electron-transport steps of ATP production, due to the leakage of electrons from mitochondria, reactive oxygen species, superoxide anion ($O_2^{-\bullet}$) and hydroxyl ($OH^{\bullet}$) radicals, are generated. These species lead to the production of hydrogen peroxide ($H_2O_2$), from which further hydroxyl radicals are generated in a reaction that seems to depend on the presence of $Fe^{2+}$ ions [7]. Free radicals have both beneficial and harmful actions (reviewed in [4]). They are needed for signal-transduction pathways that regulate cell growth [8,9], reduction-oxidation (redox) status [7], and as a first line of defense against infections by polymorphonuclear leukocytes [4]. On the other hand, excessive amounts of free radicals can start lethal chain reactions, which can inactivate vital enzymes, proteins and other important subcellular elements needed for cell survival and lead to cell death [4,10–12]. Thus, free radicals are functionally like a double-edged sword.

## LIPID PEROXIDATION IN TUMOR CELLS

It is generally believed that there is an inverse relationship between the concentrations of lipid peroxides and the rate of cell proliferation, i.e. the higher the rate of lipid peroxidation in the cells the lower the rate of cell division (reviewed in [13]). This is supported by the observation that tumor cells are more resistant to lipid peroxidation than normal cells [14,15]. Studies directed at eliciting the relationship between tumor mitochondrial membrane peroxidation and tumor cell proliferation revealed that lipid peroxidation decreases with increasing growth rate [16]. Indeed, it was shown that in hepatomas, the higher the growth rate of the tumor, the lower the microsomal phospholipid content and the degree of fatty acid unsaturation [13,17]. More specifically, examination of fatty acyl compositions of phospholipids derived from mitochondria and microsomes isolated from Morris hepatoma with slow (9618A), intermediate (7794A) and fast (7777) growth rates showed that the faster the growth rate of the tumor, the larger the sum of the amounts of 16:1, 18:1 and 18:2 fatty acids and the smaller the sum of the contents of all polyunsaturated fatty acids with four or more double bonds per molecule [18]. The former content was generally higher and the polyunsaturated fatty acid contents lower in hepatoma organelles than in the corresponding host liver organelles. For example, the 18:0/18:1 ratio observed in hepatoma organelles was always significantly less than in host or normal control organelles. In addition, the reduced rates of lipid peroxidation observed in Yoshida hepatoma cells and their microsomes when compared with appropriate control tissue (normal liver tissue) under the same pro-oxidant conditions was found to be due to the much reduced levels of NADPH-cytochrome c reductase and the NADPH-cytochrome P-450 electron transport chain in the Yoshida hepatoma cells [19]. Cheeseman et al. [20] have also reported that in the Novikoff hepatoma cells, the low rate of lipid peroxidation seems to be due to a combination of factors: low levels of polyunsaturated fatty acids and cytochrome P-450 and elevated levels of lipid-soluble anti-oxidant alpha-tocopherol. This is supported by the observation that tumor plasma membranes tested for their abilities to undergo lipid peroxidation using xanthine and xanthine oxidase as the free radical generating system, had extremely low rates of malondialdehyde accumulation and LOOH was practically undetectable in the hepatoma cell membranes compared to the normal rat liver membranes [21]. Such a high degree of resistance to peroxidation in the tumor cells has been attributed to a marked decrease in lipid content [21]. These results are very similar to those obtained by Cheeseman et al. [20]. In our studies, it was observed that incubation of cells with PUFAs augmented free radical generation and formation of lipid peroxidation products selectively in the tumor cells compared to normal cells. This increase in free radical generation and lipid peroxidation occurred despite the fact that the uptake of fatty acids was at least 2 to 3 times higher in the normal cells compared to tumor cells [22,23]. Based on these studies, it can be suggested that there is a close correlation between the rate of lipid peroxidation and degree of malignancy deviation of the tumor cell, and the susceptibility of the tumor cell to free radical induced cytotoxicity, viz: the higher the degree of malignant of the tumor cell, the lower the rate of lipid peroxidation and higher the degree of susceptibility to free radical-induced toxicity (see below). It may also be mentioned here that resistance to lipid peroxidation appears to occur at the premalignant stage of the carcinogenic process, since administration of diethylnitrosamine and 2-acetylaminofluorene leads to inhibition of peroxidation in normal liver and in preneoplastic nodules as well as in the neoplasms that result from this treatment [24].

The low content of PUFAs in the tumor cells can be attributed to the loss of or decreased activity of δ-6- and δ-5-desaturases [25–27]. This results in decreased metabolism of dietary linoleic acid (LA, 18:2, n-6) and alpha-linolenic acid (ALA, 18:3 n-3) to longer chain fatty acids such as gamma-linolenic acid (GLA, 18:3 n-6), dihomo-gamma-linolenic acid (DGLA, 20:3 n-6), arachidonic acid (AA, 20:4 n-6) and eicosapentaenoic acid

(EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) respectively. In addition, tumor cells also have elevated levels of lipid-soluble anti-oxidant alpha-tocopherol [20]. For instance, poorly differentiated, fast-growing hepatomas have a microsomal vitamin E to PUFA ratio markedly higher than the corresponding normal liver membranes, as in the highly differentiated, slow-growing hepatomas this ratio is consistently lower (reviewed in [13–16,20]). This higher the vitamin E to PUFA ratio in rapidly growing tumors is due to markedly decreased content of PUFA, while the vitamin E quantitated on a per mg protein basis is virtually unchanged. On the other hand, tumor cells have low or almost no superoxide dismutase (SOD), glutathione peroxidase and catalase enzymes [28–30]. Hence, it is possible that the relatively high content of vitamin E contributes to the low rate of lipid peroxidation observed in the tumor cells (reviewed in [13]). Thus both substrate (i.e. PUFAs) deficiency and a relatively high content of vitamin E may be responsible for the low rate of lipid peroxidation seen in various tumor cells.

## SUPEROXIDE DISMUTASE, FREE RADICALS AND TUMOR CELLS

There are two forms of superoxide dismiuase (SOD) in human cells-a mitochondrial isoform (manganese-containing SOD, MnSOD) and a copper-zinc containing SOD (CuZnSOD). CuZnSOD is primarily localized in the cytosol in mammalian cells, although some may be present in the nucleus, mitochondrial intermembrane space, lysosomes and peroxisomes. Huang et al [31] showed that transgenic mice lacking MnSOD do not survive due to severe lung damage and neurodegeneration. On the other hand, phenotypic defects in CuZnSOD-negative knockout mice are more subtle [31,32] and do not result in death. But both types of SOD are essential for healthy aerobic life.

Superoxide and other free radicals can cause cell death by apoptosis. The generation of excess radicals or severe stress can produce necrosis [32,33]. Most other death stimuli also increase formation of free radicals and cause death of the cells by apoptosis. Free radical scavengers often, but not always, delay apoptosis [33,34]. For example, edelfosine (1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine), a membrane-targeting anti-cancer ether lipid drug, induced cytotoxicity to human leukemia cells (HL-60 and K562) correlating with the production of free radicals [35]. Hepatocyte growth factor (HGF), which can suppress the growth of sarcoma 180 and Meth A cells, enhanced the generation of intracellular reactive oxygen species as judged by flow cytometric analysis using 2',7'-dichloro-fluorescein diacetate [36]. N-acetylcysteine, a precursor of glutathione and an intra-cellular free radical scavenger as well as the reduced form of glutathione, prevented HGF-suppressed growth of the tumor cells. This lends support to the involvement of free radicals in the growth suppressive action of HGF. Tumor necrosis factor (TNF)-induced tumor cell death and host toxicity can be related to its ability to induce the production of free radicals [37]. High intracellular glutathione level induce tumor cell resistance to recombinant human TNF (rhTNF), whereas low glutathione levels enhanced sensitivity to rhTNF. Conversely, pretreatment of the tumor-bearing hosts with DL-buthionine-(S, R)-sulfoximine, an inhibitor of GSH biosynthesis, resulted in an increased sensitivity of tumor cells to rhTNF [37]. Induction of apoptosis by arachidonic acid (AA) in human retinoblastoma cells and other polyunsaturated fatty acids (PUFAs) were also found to be accompanied by increased formation of the lipid peroxidation end products, such as malondialdehyde and 4-hydroxynonenal, suggesting a role for free radicals [22,38].

SODs convert superoxide radical ($O_2^{-\bullet}$) into hydrogen peroxide ($H_2O_2$) and oxygen ($O_2$). Bize et al [39] showed that both total SOD and MnSOD specific activities were lower in the tumor cell homogenates compared to normal liver: the lowest activity was associated with the fastest growing tumor. MnSOD activity was decreased in the fast- and medium-growth rate hepatomas but was slightly increased in the tumor with the slowest growth rate compared to normal liver. This suggests that decreased MnSOD specific activity is not a characteristic of all tumors. In addition, it was also observed that antimycin A (an inhibitor of the electron transport chain) stimulated the production of $O_2^{-\bullet}$ in normal rat liver and slow-growth-rate tumor cells but not in the submitochondrial particles of fast-growth-rate tumor cells [39], lending support to the concept that the rate of lipid peroxidation is generally low in tumor cells.

In a study designed to determine the factors that contribute to the decreased rates of lipid peroxidation under different pro-oxidant conditions in Novikoff tumor cells, Cheeseman et al. [40] observed a significant decrease in the PUFA content of Novikoff cells or Novikoff microsomes, especially arachidonic acid (AA, 20:4 n-6) and eicosa-pentaenoic acid (EPA, 20:5 n-3). There was also a marked reduction in NADPH-cytochrome c reductase. A substantial increase in alpha-tocopherol relative both to total lipid and to methylene-interrupted double bonds in fatty acids was also found. Thus both Novikoff rat liver and Yoshida liver tumors showed similar biochemical changes and decreased rates of lipid peroxidation [19,40]. However, it should be noted here that although many malignant cell lines show low SOD activity, reduced SOD activity has not been found in all tumors. In fact, in some cases SOD activities were found to be higher in certain tumors than in normal tissues [41,42], and paradoxically in some human gliomas the malignant phenotype could be suppressed by overexpressing MnSOD [43]. In gastric and colorectal adenocarcinomas overexpression of MnSOD may correlate with aggressiveness of the tumor [44]. Similarly, in both oesophageal and gastric cancers, the levels of MnSOD mRNA was significantly elevated compared to normal tissue [45]. But it is not known whether there is any relationship between the aggressiveness of tumors with the levels of SOD. In some studies it was observed that the activities of MnSOD and CuZn SOD varied greatly among both human and rat glioma cells [46]. On the other hand, Huang et al. [47] noted that the lower levels of MnSOD in SV40-transformed cells

was related to increased cytosine methylation of the SOD2 intron region (the SOD2 gene contains a large CpG island spanning greater than 3.5 kb that starts near the 5'edge of the promoter and extends into intron 2).

The SH groups of the caspases are essential for their catalytic activity. On exposure to free radicals these SH groups may be inactivated. In M14 melanoma cells, $O_2^{-\bullet}$ and $H_2O_2$ may, in fact, seem to promote tumor cell survival due to the inactivation of caspases [48]. Similar to superoxide anion and $H_2O_2$, NO (nitric oxide) also may have a role both in cancer suppression and progression [6]. But, in general, it can be suggested that majority of the tumors have low SOD concentrations, relatively high amounts of vitamin E, low PUFA content and lipid peroxides, which may render them more susceptible and sensitive to free radical attack. Perhaps, the best strategy to make use of free radical toxicity to tumor cells would be to devise methods or chemicals that can lower SOD, catalase and glutathione and vitamin E concentrations, and at the same time enhance free radical generation and lipid peroxidation process in the tumor cells.

## FREE RADICALS, LIPID PEROXIDES AND CELL PROLIFERATION

If it is true that oxidant stress and the anti-oxidants play a significant role in the control of tumor cell proliferation and survival, what could be their role in normal cell proliferation and apoptosis? It is generally believed that there is a negative correlation between cell proliferation and lipid peroxidation. For example, cells that proliferate rapidly such as spermatozoa show low rates of lipid peroxidation whereas neurons, which seldom divide, have high concentrations of lipid peroxides. In a series of studies, Cornwell et al. [49–51] showed that in primary cultures of smooth muscle cells, cell proliferation is controlled, at least in part, by general peroxidation reactions rather than the specific peroxidation reactions involved in prostanoid synthesis, when these cells were supplemented with various PUFAs. In particular, they observed that the inhibition of primary cultures of smooth muscle cells was related to the structures of different PUFA families decreasing in the order n-9 > n-6 > n-3. Since the inhibition of cell proliferation did not correlate with stimulated or inhibited prostaglandin synthesis in these studies, it was concluded that fatty acids themselves modulate cell proliferation. The growth inhibitory actions of PUFAs could be blocked by anti-oxidants such as vitamin E, butylated hydroxytoluene (BHT), α-naphthol, 6-hydroxy-2,5,7,8-tetramethyl-chrom-2-carboxylic acid and dipyridamole [49] indicating a role for free radicals and lipid peroxides.

Cheeseman et al. [52] observed that in the partially hepatectomised animals the period of liver regeneration is characterized by regular bursts of thymidine kinase activity. The periods of maximum thymidine kinase activity of liver miscrosomes corresponded to the reduced rates of lipid peroxidation compared to control sham-operated animals. The reduced rate of lipid peroxidation was found to be related, in part, to increased levels of the lipid-soluble anti-oxidant alpha-tocopherol at times of maximum DNA synthesis [52,53]. This led the authors to conclude that lipid peroxidation is decreased prior to cell division. With the observation that the majority, if not all, tumors have low SOD concentrations, relatively high amounts of vitamin E and low PUFA and lipid peroxide contents, tilting the cellular balance more towards higher anti-oxidant capacity may explain, in part, why tumors frequently have decreased rates of cell death and increased rates of cell proliferation. Since genetic changes resulting in loss of programmed cell death (apoptosis) are critical components of tumorigenesis, it is important to know whether there is any relationship between free radicals, lipid peroxidation and factors that contribute to or control apoptosis, such as p53, Bcl-2, caspases, telomeres and telomerases.

## FREE RADICALS, LIPID PEROXIDES, P53, CASPASES AND APOPTOSIS

Many of the gene products that appear to control apoptosis are also regulators of cell cycle progression. Thus cell cycle control and cell death appear to be linked. P53 protein is an example of a gene product that affects both cell cycle progression and apoptosis [54]. The ability of p53 overexpression to induce apoptosis may be a major reason why tumor cells, more often than not, disable p53 during the transformation process. At the same time, the same genetic changes that cause loss of apoptosis during tumor development may also result in tumor cell resistance to anti-cancer therapies that kill tumor cells by apoptosis. Hence, elucidation of the genetic and biochemical controls of these cellular processes may provide insights into induction of apoptosis and thus, hopefully, suggest new therapeutic modalities in the treatment of cancer. In view of this, it is pertinent to know whether there is any relationship between free radicals, lipid peroxides, Bcl-2, p53 and caspases, which determine ultimately whether the cell has to undergo apoptosis or survive and proliferate.

The p53 tumor suppressor is a transcription factor that regulates several gene expression pathways that function collectively to maintain the integrity of the genome. Its nuclear localization is critical to this regulation. In 1997 Martinez et al [55] reported that ionizing radiation caused a biphasic p53 translocation response: p53 entered the nucleus 1-2 hours post-irradiation, subsequently emerged from the nucleus, and then again entered the nucleus 12-24 hours after the cells had been irradiated. These changes in the subcellular localization of p53 could be completely blocked by the free radical scavenger WR 1065. It was also noted that mitomycin C and doxorubicin, two DNA-damaging agents that do not generate free radicals, caused translocation of p53 only after 12-24 hours of exposure to the drugs, and this effect could not be blocked by WR 1065. These results indicate that although all three DNA-damaging agents induced relocalization of p53 to the nucleus, only trans-localization caused by irradiation was sensitive to free radical scavenging. Thus free radicals seem to have the ability to signal p53 translocation to the nucleus.

This free radical induced translocation of p53 could be an indication that free radicals damage DNA and p53, in all probability, is involved in maintaining the integrity (by inducing DNA repair) of the genome by translocation to the nucleus. This is supported by the observation that brief exposure of oligodendroglia-type cell line (OLN 93) to $H_2O_2$ produced a marked translocation of p53 from the cytosolic to the nuclear compartment within 20 minutes of exposure [56]. By 48 hours of exposure to $H_2O_2$, nearly 60% of the cells exhibited p53 in the nuclei, at which time a large proportion of the cells underwent apoptosis as determined by DAPI nuclear staining. The genotoxic-induced p53 relocalization appeared to be cell cycle-specific, since cells in the G0/G1 stage had more abundant nuclear-associated p53 and were also more susceptible to $H_2O_2$-induced apoptosis than the cells in G1/S phase. It was also observed that genes p21 and mdm2 were upregulated following p53 nuclear translocation, and mdm2 enhancement accelerated the exit of p53 from the nucleus to the cytosol [56]. These results suggest that following exposure to $H_2O_2$, cells are induced to undergo p53-dependent apoptosis, an event that coincides with p53 nuclear translocation and is cell cycle related. Kitamura et al. [57] also reported that $H_2O_2$-induced apoptosis is mediated by p53 protein in glial cells. It was noted that in human A172 glioblastoma cells $H_2O_2$ caused cell death in a time- and concentration-dependent manner, accompanied by nucleosomal DNA fragmentation and chromatin condensation [57]. Exposure of A172 cells to $H_2O_2$ led to increased expression of p53 and enhancement in the protein levels of Bak, p21WAF1/CIP1 and GADD45 with no change in the protein levels of Bcl-2 and Bax. On the other hand, primary cultured astrocytes from p53-deficient mouse brain grew faster than wild-type and heterozygous astrocytes and were also more resistant to $H_2O_2$-induced apoptosis than wild-type and heterozygous astrocytes. These results suggest that glial proliferation and the repair of damaged DNA is regulated by p53-induced p21WAF1/CIP1 and GADD45, and that glial apoptosis caused by oxidative stress induced by $H_2O_2$ is mediated by p53. These results are similar to those reported by Uberti et al. with OLN 93 cells [56]. In summary, these results [55–57] suggest that free radicals are powerful inducers of p53 activity and that they play a role in the execution of p53-dependent apoptosis.

In this context, it is interesting to note that transformed mouse fibroblasts lacking p53 are significantly more resistant than wild-type controls to the cytotoxic effect of a number of pro-oxidants [58]. Further, it was noted that MnSOD activity was increased in liver tissue from p53-deficient mice in comparison with wild type. In addition, transient transfection of HeLa cells with p53 led to a significant reduction in steady-state MnSOD mRNA levels and enzymatic activity, suggesting that expression of the anti-oxidant enzyme MnSOD is negatively regulated by p53. Increased expression of MnSOD rendered HeLa cells resistant to p53-dependent cytotoxic treatments and, in co-transfection experiments, counteracted the growth inhibitory effect of p53 [58]. These results suggest that p53 inhibits the expression of MnSOD, a protein known to protect cells from the oxidative injury induced by various cytokines and anti-cancer drugs. Based on this [55–58], it can be suggested that normally there is a balance maintained between p53 and SOD levels and that overexpression of p53 can lead to a decrease in the levels of SOD. On the other hand, in some, if not all, tumor cells that have impaired p53 activity, the SOD activity is likely to be high, imparting increased anti-oxidant capacity to these cells. This suggests that p53 has a pro-oxidant type of activity. Thus, when tumor cells are exposed to free radicals such as $O_2^-$, $H_2O_2$ or NO, SOD present in the cells/tissues is not only utilized to quench these free radicals but, will also lead to an increase in the expression of p53 due to free radical induced stress [56,57]. This in turn suppresses the expression of SOD, tilting the balance more towards a pro-oxidant state. The increase in the pro-oxidant state can induce apoptosis. Further, both free radicals and lipid peroxides can also induce damage to a variety of enzymes, proteins and deplete ATP levels in the cells and thus cause cell death [13,59]. Another mechanism by which free radicals, especially $H_2O_2$, can cause ATP depletion in the cells is by activating PARP (poly-ADP-ribose-polymerase), the substrate of caspase-3 [38], though there is some controversy about this [60]. It should be remembered that $H_2O_2$ induces apoptosis at low concentrations, but at higher concentrations causes necrosis. Necrosis is known to occur if the oxidative stress is severe [6,33]. Hence, the final effect of $H_2O_2$ on tumor cell death, whether it is by apoptosis or by necrosis, is dependent on the concentration of $H_2O_2$ present.

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## OXIDANT STRESS AND TELOMERE

Telomeres of human somatic cells shorten with each cell division but are stabilized at constant length in tumors by the enzyme telomerase [61]. Recently, it has been demon-strated that oxidative stress is a main reason for telomere shortening. This is supported by the observation that $H_2O_2$ plus Cu(II) caused predominant DNA damage at the site of 5'-GGG-3' in the telomere sequence [62]. Further, $H_2O_2$ plus Cu(II) induced 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxodG) formation in telomere sequences more efficiently than in non-telomere sequences. NO plus $O_2^{-\bullet}$ efficiently caused base alterations at the 5' site of 5'-GGG-3' in the telomere sequence [62]. From these results, it is evident that free radicals play an important role in telomere shortening. The preferential vulnerability of telomeres to oxidative stress was also reported by von Zglinicki et al [63]. It was observed that treatment of non-proliferating human MRC-5 fibroblasts with $H_2O_2$ increased the sensitivity to S1 nuclease in telomeres preferentially and accelerated the shortening of telomeres by a corresponding amount as soon as the cells were allowed to proliferate. On the other hand, a reduction in the activity of intracellular peroxides using spin trap, alpha-phenyl-t-butyl-nitrone reduced the telomere shortening rate and increased the replicative life span. It was also noted that the telomere shortening rate and the rate of replicative aging can be either accelerated or decelerated by changing the amount of oxidative stress [63], indi-

cating that free radical mediated telomere damage contributes to telomere shortening.

## OXIDANT STRESS, BCL-2 AND APOPTOSIS

One of the factors that can oppose the pro-oxidant action of p53 is Bcl-2. It has been shown that Bcl-2 protein has anti-oxidant action [64] and the ability to suppress SOD activity. This suggests that a balance could normally exist between the levels of p53, which induces a pro-oxidant status in cells, and the expression and anti-oxidant activity of Bcl-2. Thus, it is possible that increased expression of p53 expression such as during exposure to radiation and free radicals, can lead to inhibition of Bcl-2 expression and a further increase in free radical generation and apoptosis. This is supported by the observation that Bcl-2 can be down-regulated by p53 [65].

Although the anti-apoptotic potential of Bcl-2 is well established, the exact mechanism of its action is not clear. Recent studies suggested that the action of Bcl-2 depends on its phosphorylation. Halder et al. [66] reported that Bcl-2 is phosphorylated in lymphoid cells, especially on serine residues. The phosphatase inhibitor okadaic acid and chemotherapeutic drug taxol can phosphorylate Bcl-2 in lymphoid cells. Under these conditions, both taxol and okadaic acid induced apoptosis in lymphoid cells. Thus, phosphorylation of Bcl-2 prevented its ability to interfere with apoptosis [66]. Bcl-2 is known to block lipid peroxidation that is known to occur with apoptosis [67]. Okadaic acid treatment of lymphoid cells, which induces apoptosis due to phosphorylation and inactivation of Bcl-2, could no longer prevent peroxidation of cis-parinaric acid [66]. This suggested that Bcl-2 expression and lipid peroxidation are closely related. It is possible that whenever there is an increase in the expression of Bcl-2, lipid peroxidation does not occur (or Bcl-2 expression prevents lipid peroxidation) and apoptosis is blocked. On the other hand, following an apoptotic signal, progressive lipid peroxidation could occur in the cells, whereas overexpression of Bcl-2 suppresses lipid peroxidation [67]. Thus, there appears to be a close association between lipid peroxidation, the action of Bcl-2 and apoptosis [68]. In this context, it is interesting to note that we have shown that when tumor cells are treated with PUFAs, in addition to an enhancement in the formation of lipid peroxides, there was also an increase in protein phosphorylation [69]. Thus, methods designed to enhance lipid peroxidation in the tumor cells may lead to phosphorylation of Bcl-2 and a reduction in its anti-apoptosis potential, and hence, apoptosis would be stimulated. This possible interaction between lipid peroxidation and Bcl-2 is understandable since Bcl-2 is localized at intracellular sites of oxygen free radical generation including mitochondria, endoplasmic reticulum and nuclear membranes.

There seems to be a close interaction between pro-apoptotic and anti-apoptotic events in the cells. Ultimately, it is the balance between these various signals and chemicals that will determine whether the cell will undergo apoptosis or survive and proliferate. The optimum expression of Bcl-2, and the presence of adequate concentrations of anti-oxidants such as SOD, catalase, glutathione, and vitamin E prevent apoptosis, whereas activation of p53, excess production of free radicals and an increase in the levels of lipid peroxides in the cells would trigger apoptosis. In this balance between pro-apoptotic and anti-apoptotic signals and chemicals, the interactions among them are interesting: pro-apoptotic signal p53 regulates Bcl-2 and SOD levels, anti-apoptotic signal Bcl-2 controls free radical generation and lipid peroxidation process, whereas lipid peroxidation itself can promote Bcl-2 phosphorylation and thus, inactivate Bcl-2.

There is also an interesting relationship between Bcl-2 and free radicals. In Burkitt's lymphoma cells, increased availability of mitochondrial NAD(P)H was detected in Bcl-2 expressing cells that was correlated with an increased constitutive mitochondrial production of $H_2O_2$ [70]. Although production of $H_2O_2$ was increased by TNFα in Bcl-2- negative lymphoma cells commensurate with the early phases of apoptosis, this increase was not seen in Bcl-2 expressing cells. This suggests that Bcl-2 appears to allow cells to adapt to an increased state of oxidative stress, fortifying the cellular anti-oxidant defenses [70].

In view of the relationship between cellular PUFA and anti-oxidant content, expression of p53, inactivation of Bcl-2 by phosphorylation and lipid peroxides and the increased sensitivity of tumor cells to oxidant stress compared to normal cells, it appears that strategies that augment free radical generation specifically in tumor cells can induce their apoptosis. 2-methoxy-oestradiol (2-ME), PUFAs and thalidomide seem to possess such an ability. Although cytokines, such as TNF, ILs (interleukins) and some anti-cancer drugs, radiation and protoporphyrin derivatives (used in photodynamic therapy), do have the capacity to augment free radical generation and lipid peroxidation process in the tumor cells, they have many undesirable side-effects. 2-ME, PUFAs and thalidomide seem to have few side-effects, significant cytotoxic action on tumor cells and anti-angiogenic properties, and have the ability to augment free radical generation specifically in the tumor cells. Though 2-ME is yet to be tested in the clinic, its in vitro and in vivo activities are promising. On the other hand, PUFAs, especially n-3 fatty acids, showed promise in animal tumor models and GLA (an n-6 fatty acid) was found to regress human gliomas with few side-effects. Thalidomide is now being tested in patients with multiple myeloma.

## 2-METHOXYOESTRADIOL INHIBITS SOD AND AUGMENTS FREE RADICAL GENERATION

2-methoxyoestradiol (2-ME) is a natural metabolite of estradiol, which has been shown to have cytotoxic effects on the breast cancer cells, such as MCF-7 cells [71]. Studies have shown that 2-ME interferes with spindle formation, DNA synthesis and thus can inhibit the growth of Chinese hamster V79 cells *in vitro* [72]. In the

MCF-7 cells, 2-ME has been shown to bind to the colchicine-binding site of the tubulin filaments resulting in tubulin polymerization or alterations in the stability of microtubules [73]. In addition, 2-ME also inhibits endothelial cell migration and angiogenesis *in vitro,* which contributes to its growth inhibitory action on tumor cells both *in vitro* and *in vivo* [74]. Mukhopadhyay and Roth [75] showed that 2-ME induces accumulation of p53 protein and thus causes apoptosis of tumor cells. On the other hand, Huang et al [76] observed that 2-ME induced typical apoptotic morphology in human leukemic cells with different p53 genotypes *in vitro*. In a further extension of these studies, Huang et al. [76] also showed that 2-ME inhibited SOD leading to free radical-mediated damage to mitochondrial membranes and the release of cytochrome c. Because $O_2^{-\bullet}$ also gives rise to hydroxyl radicals and cause DNA damage, it was suggested that this may explain the accumulation of p53 in 2-ME-treated cells since p53 has pro-oxidant actions. In view of the interactions between p53, Bcl-2, lipid peroxidation and SOD, it is possible that 2-ME may enhance the concentrations of lipid peroxides (since free radical generation is increased by 2-ME), and suppress Bcl-2 expression. But this needs to be more carefully established. It is also not known whether vitamin E, a potent anti-oxidant, can interfere with the cytotoxic action of 2-ME. 2-ME is not known to bind to the estrogen receptor, but it is possible that other structurally related analogues may do so. The safety and efficacy of long-term use of 2-ME in humans is also not certain. Since the ability of $O_2^{-\bullet}$ to induce apoptosis may vary among different cell types [77], it may be necessary to combine 2-ME with other modalities of therapy that produce free radicals to augment its anti-cancer activity. Such studies have yet to be performed.

## POLYUNSATURATED FATTY ACIDS, CELL PROLIFERATION, AND LIPID PEROXIDATION

Several laboratories have reported that PUFAs (especially GLA, AA, EPA and DHA; LA and ALA are not included under PUFAs in the present discussion) when used at appropriate doses inhibit proliferation of cells *in vitro,* where as anti-oxidants block this inhibitory action [49–51]. Prostaglandins (PGs) derived from PUFAs can also inhibit the proliferation of human and animal tumor cells in vitro [78–80]. When used at appropriate concentrations, long chain unsaturated fatty acids, such as GLA, AA, EPA and DHA, were found to be toxic to tumor cells with little or no effect on the survival of normal cells *in vitro* [81–86]. Seigel et al. [87] and Tonai and Morgan [88] showed that unsaturated fatty acids have cytotoxic effects on tumor cells. This selective tumoricidal action of fatty acids was not blocked by cyclo-oxygenase and lipoxygenase inhibitors, suggesting that prostaglandins and leukotrienes do not participate in this process [81–85]. In an extension of these studies, it was reported that anti-oxidants such as vitamin A, vitamin E, BHA and BHT can completely inhibit the tumoricidal action of PUFAs (reviewed in [13]), indicating that free radicals and lipid peroxidation products could be the mediators of their (PUFAs) cytotoxic action. This is supported by the observation that GLA, AA and EPA-treated tumor cells but not normal cells produce, at least, a 2–3 fold increase in free radicals and lipid peroxidation products [22,23,83–85]. Tumor cells are susceptible to the cytotoxic action(s) of free radicals and lipid peroxides (as discussed above and in refs [89,90]). Tumor cells also have decreased activities of NADPH cytochrome C reductase, cytochrome P-450 and low amounts of PUFAs [12,13,19–21,40,91,92]. Supplementation of PUFAs to tumor cells induced generation of excess of free radicals and lipid peroxidation process. These results suggest that the low rates of lipid peroxidation observed in tumor cells are, at least in part, due to substrate deficiency (mainly PUFAs) and to a relative increase in their anti-oxidant content [19–21,25–30,91–93]. Since there is a direct correlation between the rate of lipid peroxidation and the degree of deviation in hepatomas [13–16,20,94] and as the rate of lipid peroxidation is low in several tumors, it is suggested that lipid peroxidation might act as a physiological inhibitor of mitosis and might regulate cell multiplication [49–53,95,96].

The cytotoxic action of PUFAs was seen irrespective of the form in which these fatty acids were delivered to the tumor cells. For instance, in our studies free fatty acids, methyl or ethyl esters of fatty acids were used [81–86]. On the other hand, Jett and Alving [97] used liposomes containing highly purified phosphotidylinositol of plant origin. Jett et al. [98] have demonstrated that liposomes composed of synthetic forms of phosphotidylinositol (PI) can also be metabolized by the tumor cells leading to the release of free linolenic and arachidonic acids due to the activation of phospholipase $A_2$. Based on their studies Jett et al. [98] suggested that it is the free acids that could be responsible for the cytotoxicity of PI. Shimura et al. [99,100] showed that the viability of transformed rat fibroblasts was markedly impaired by dilinoleoyl glycerol, due to the release of LA by phospholipase $A_2$, and that this cytotoxicity can be markedly reduced by the simultaneous administration of anti-oxidants and leukotriene inhibitors. Palozza et al. [101] also reported that EPA inhibited the growth of WiDr adenocarcinoma cells in a dose- and time-dependent manner and that this inhibitory effect was associated with a remarkable increase in the levels of malondialdehyde in EPA-treated cells, which is an indication of increase in lipid peroxidation. On the other hand, β-carotene antagonized the effects of EPA on colon cell growth and lipid peroxidation, indicating that there is a close relationship between growth and lipid peroxidation.

The role of free radicals and lipid peroxidation in the tumoricidal action of fatty acids is further supported by the fact that free radicals can cause DNA strand breaks [102], whereas polyamines, which are produced in large amounts by tumor cells, have a radical scavenging action and prevent lipid peroxidation and protect against ozone damage [103,104]. Further, PUFAs induce apoptosis in tumor cells [68,105–107], which in itself is an indication of damage to DNA. Colon adenocarcinoma cells that over-express cyclo-oxygenase-2 (COX-2) enzyme also overexpress another arachidonic acid-utilizing enzyme, fatty acid-CoA ligase (FACL) 4 [107]. Both exogenous AA and triacsin C, a FACL

inhibitor, caused apoptosis in colon cancer cells. In addition, indomethacin and sulindac, which are COX-2 inhibitors, significantly enhanced the apoptosis-inducing effect of triacsin C. On the other hand, when these colon cancer cells were engineered to over-express COX-2 and FACL4, this resulted in an inhibition of apoptosis and a decrease in the cellular level of AA was noted [107]. These non-steroidal anti-inflammatory drugs (NSAIDs) also inhibited the expression of the anti-apoptotic protein Bcl-XL and enhanced the levels of BAX, the prototypic death-promoting member of the Bcl-2 family, resulting in an altered ratio of BAX to Bcl-$X_L$ [108]. This suggests that BAX has an important role in the apoptotic process. It is also interesting that cell death caused by TNFα is associated with release of endogenous AA [107,109], whereas TNFα-induced apoptosis can be prevented by the removal of unesterified AA [107]. Thus the cellular level of unesterified AA may be a general mechanism by which apoptosis is induced in colon and other tumor cells. Since other PUFAs, such as GLA, DGLA, EPA and DHA, can also induce apoptosis of tumor cells, it is likely that methods designed to enhance cellular content of unesterified PUFAs may trigger apoptosis in the tumor cells [68]. This may also explain the beneficial action of EPA- and DHA-rich fish oils in the prevention of colon cancer [110,111]. Further, tumor cells exposed to PUFAs show low levels of various anti-oxidants [85,86], which may cause an increase in oxidant stress and an enhancement in cytotoxicity. In addition, n-3 PUFAs are capable of suppressing carcinogen induced *ras* activation [112], Bcl-2 expression, and inhibit the activity of cyclo-oxygenase enzyme. Thus PUFAs have several activities that contribute to their anti-cancer actions [68].

There appear to be many similarities in the actions of 2-ME and PUFAs on tumor cells: both are naturally occurring endogenous substances, augment free radical generation, suppress SOD levels, enhance p53 expression and induce apoptosis [68,76,113].

## Anti-cancer actions of thalidomide and free radicals

Thalidomide causes stunted limb growth during embryogenesis. It exerts anti-angiogenic effects on the development of capillary structures. In view of its anti-angiogenic actions, it is now being tried in the treatment of erythema nodosum leprosum, systemic lupus erythematosus, Kaposi's sarcoma and multiple myeloma [114]. Thalidomide seems to have the ability to reduce the activity of the inflammatory cytokine, TNFα, and inhibit angiogenesis induced by basic fibroblast growth factor and vascular endothelial growth factor [114–116]. It is interesting that thalidomide enhanced the generation of hydroxyl radicals in murine embryonic stem cells [117]. Parman et al. [118] showed that in rabbits thalidomide-induced embryonic DNA oxidation and teratogenicity could be abolished with the free radical spin trapping agent alpha-phenyl-N-t-butylnitrone (PBN). Thalidomide also enhanced superoxide anion release from human polymorphonuclear and monocyte leukocytes [119]. This suggests that thalidomide exerts its anti-angiogenic properties via the generation of free radicals that impair vasculogenesis and angiogenesis. If this is true, does this mean that all anti-angiogenic compounds augment free radical generation or that agents that augment free radical generation have anti-angiogenic action?

## Free radicals and angiogenesis

2-ME is known to bind to CuZnSOD and cause inhibition of its activity and thus causes accumulation of $O_2^{-\bullet}$. This ultimately results in apoptosis of tumor cells [76]. In addition, 2-ME also has potent anti-angiogenic action [74]. But studies have not been performed to see whether there is any correlation between its anti-angiogenic action and ability to enhance free radicals. In a similar fashion PUFAs, which also have the ability to augment free radical generation in tumor cells [83], have been shown to possess anti-angiogenic action. Cai et al [120] showed that in a rat aortic ring assay and in an *in vitro* tube formation assay of human vascular endothelial cells, GLA suppressed angiogenesis in a concentration dependent manner. Furthermore, a significant reduction of the motility of vascular endothelial cells by GLA was seen as assessed in phagokinetic and endothelial wounding assays. DHA, which can suppress the growth of tumors both *in vitro* and *in vivo,* also showed anti-angiogenic activity [121]. Thalidomide, which inhibits tumor cell proliferation and enhances free radical generation, also has anti-angiogenic activity [114–116]. These results suggest that there may be a close association between free radical generation and angiogenesis. In other words, it is proposed that the anti-angiogenic action of 2-ME, thalidomide and PUFAs may be ascribed to their ability to trigger free radical generation. But, it is not yet known whether other anti-angiogenic agents such as endostatin and angiostatin can also induce free radical generation.

## Conclusions and clinical implications

It is evident from the preceding discussion that there is a close interaction between free radicals, lipid peroxidation and apoptosis. In general, but with some exceptions, tumor cells seem to have relatively higher anti-oxidant capacities and low rates of lipid peroxidation which may favour tumor cell proliferation. High-fat, n-6 fatty acid-rich diets were also associated with a relatively poor prognosis in breast cancer patients. In the nude mouse model, these diets enhanced breast cancer progression, whereas n-3 fatty acids (rich in EPA and DHA) exerted suppressive effects [122,123]. In cell culture studies, LA-stimulated growth of tumor cells [124]. These studies led to the suggestion that n-6 fatty acids, in general, augment tumor growth. It may be mentioned here that majority of the studies that refer to the effects of n-6 fatty acids on tumor growth used oils, which were rich in LA. These LA-rich oils did not contain any GLA or AA, which are also n-6 fatty acids, but are metabolites of LA. On the other hand, studies performed with oils, which contain both LA and GLA (such as primrose oil) gave opposite results, namely suppression of tumor growth [125–127]. This suggests that LA may promote whereas other n-6 fatty acids such as GLA,

DGLA and AA suppress tumor growth [110,128]. So one has to be cautious when interpreting results obtained with LA alone and such results should not be generalized to all n-6 fatty acids. This is supported by the observation of many laboratories that GLA, salts of GLA and a chemical formulation containing both GLA and EPA inhibit tumor growth *in vitro* and *in vivo* [119–134].

Both *in vitro* and *in vivo* studies generally led to the belief that PUFAs inhibit or damage tumor cells by the generation of free radicals and lipid peroxides, but it may be noted here that they may also do so indirectly by modulating the body's immune system [135] and inhibiting adhesion of tumors with endothelium and other cells [136,137]. It was reported that PUFAs enhance human breast cancer cell adhesion to collagen IV by selectively activating specific PKC (protein kinase C) isozymes, which leads to the activation of $\beta_1$ integrins [138]. It was also reported that p38 MAP kinase may have a role in the regulation of adhesion of breast cancer cells to collagen type IV [139]. This may have relevance to the role of PUFAs in the invasion and metastasis of cancer cells. Bell et al [140] observed that low concentrations of GLA (<100 microM) increased growth and invasion of glioma cells whereas high-dose (>100 microM) significantly impaired their growth. In view of this, it is important to know the dose at which the fatty acid that is being used is most effective against the type of tumor against which it is being used. It is possible that different tumor cells may have different responses to various PUFAs. Diggle et al [141] showed that normal regenerating uroepithelial cells are sensitive to the growth inhibitory effects of n-3 and n-6 PUFAs (especially GLA, EPA and DHA), both acting via p53-independent mechanisms, and cells with genomic instability may acquire resistance. Hence, they advocated caution in the use of PUFAs as chemotherapeutic adjuncts. But, several other studies suggested that GLA and other PUFAs enhance tumor cell chemosensitivity [129,132,133,142–144]. PUFA incorporation into the cellular membranes of tumor cells may cause a shift in the uptake and intracellular distribution of chemotherapeutic drugs and this rendered them susceptible to the cytotoxic action of conventional drugs and radiation or even reverse tumor cell drug resistance [145–147].

PUFAs also affected G-protein–mediated signal transduction [148] and are able to mobilize $Ca^{2+}$ from intracellular stores [149], which may induce apoptosis [150]. PUFAs activate PKC and enhance NADPH oxidase in macrophages [151]. PUFAs increase $O_2^{-\bullet}$ by more than 50% in murine lymphoid tumor cells [152] and in other type of tumor cells [12,22,23,83–86] which may be responsible for their tumoricidal action. There is evidence to suggest that EPA decreased Bcl-2 while increasing Bax in tumor cells [153]. In addition, DHA increased p27, inhibited cyclin-associated kinase, reduced pRb phosphorylation and cause some melanoma cells to undergo apoptosis [154,155]. There is evidence to suggest that PUFAs (especially EPA) inhibit cell division by inhibiting translation initiation, preferentially reducing the synthesis and expression of G1 cyclins both *in vitro* and *in vivo* [156]. This is an interesting since overexpression of cyclin A1 is known to be involved in the development of acute myeloid leukemia [157] and decline in cyclin D1 levels induced by nitric oxide is responsible for cytostasis and arrest of tumor breast cancer cell line MDA-MB-231 growth in the $G_1$ phase of the cell cycle [158]. In addition, free radicals, especially $H_2O_2$, have been shown to directly activate purified heterodimeric $G_i$ and $G_0$ (small G proteins) [159], which are important signaling molecules. Thus, PUFAs are able to target several intracellular second messenger molecules and genes either directly or by inducing the production of and free radicals and this may ultimately induce tumor cell death.

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The proposal that the free radical system can be used to kill tumor cells selectively need not necessarily contravene the belief that free radicals damage DNA and thus participate in mutagenesis and carcinogenesis [160]. It appears as though the actions of free radicals on normal and tumor cells are diametrically opposite; viz. when free radicals attack normal cells, DNA damage can occur, leading to the development of tumors, whereas when the same free radicals are produced in excess in tumor cells, there is a totally unexpected but highly beneficial action, namely elimination of those cells. In the latter instance also, the mechanism of action appears to be damage to DNA, but the end results differ depending on whether the target is normal or tumor cells [161,162]. This idea is supported by Dormandy [163], who proposed that 'excessive peroxidation and free radical activity may need inhibiting' especially in the normal cells to prevent carcinogenesis, 'inadequate peroxidation and free radical activity need boosting' to kill tumor cells, and 'normal peroxidation and free radical activity should be left alone'.

Furthermore, both free radicals and lipid peroxides seem to suppress Bcl-2 and enhance p53 expression, induce telomere shortening and thus arrest tumor cell growth and cause apoptosis (see Figure 1). Thus, inducing the generation of adequate amounts of free radicals and consequent lipid peroxidation appears to be critical to trigger tumor cell apoptosis. Both 2-ME and PUFAs seem to have this unique property [76,82,83]. In addition, 2-ME and PUFAs (especially GLA and DHA) have anti-angiogenic action as well. But, the exact mechanism by which PUFAs produce their anti-angiogenic action is not known. Recent studies showed that tumor suppressor gene PTEN regulates tumor-induced angiogenesis [164] and that phosphoglycerate kinase increases plasma levels of angiostatin and decreases tumor vascularity and rate of tumor growth [165]. It is possible that PUFAs may modulate the expression of PTEN and action of phosphoglycerate kinase. But, this remains to be established. This anti-angiogenic action of PUFAs facilitates their anti-cancer action. Further some PUFAs (especially EPA) have anti-cachectic effect [166] which may be an added benefit in cancer. Though 2-ME has not yet been tested in humans, it is now undergoing phase 1 clinical evaluation. On the other hand, in a limited clinical study we have shown that intra-tumoral

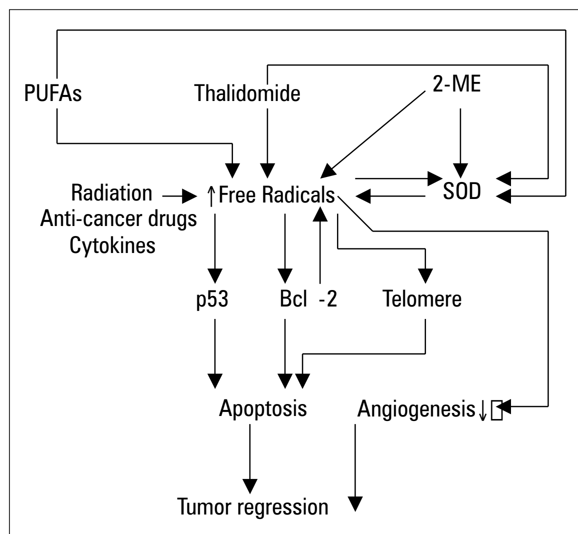


**Figure 1.** Scheme showing possible interaction between PUFAs, thalidomide, 2-ME, radiation and anti-cancer drugs and free radicals, angiogenesis, apoptosis, and tumor regression.

injection of GLA can regress human brain gliomas without any significant side-effects [167–169]. GLA when injected into the normal brain tissue of healthy dogs did not show any side-effects, suggesting that it is non-toxic to normal cells. In a preliminary study it was found that GLA induced remission of Hodgkin's disease which was resistant to chemotherapy [170]. These results indicate that PUFAs may benefit some patients with cancer. Obviously more studies are needed to confirm these studies. Perhaps methods should be devised to selectively deliver PUFAs to tumor cells so that they can exert their tumoricidal action. This is so because PUFAs can bind to albumin and other proteins and thus render them unavailable to the tumor cells when they are given orally or parenterally [128,130,131,171]. Therefore the methods designed to selectively deliver PUFAs, thalidomide and 2-ME and other anti-cancer drugs are important so that only tumor cells are eliminated and the side-effects or actions on normal cells are minimized. It is also important to study whether a combination of radiation and the currently available chemotherapeutic agents, such as doxorubicin, cis-platinum, etc. along with PUFAs, 2-ME and/or thalidomide would be more beneficial in eliminating tumor cells [132–134,172,173]. Some *in vitro* studies do suggest that this could indeed be the case. Thus methods designed to selectively enhance free radical generation and lipid peroxides and suppress the anti-oxidant defenses in the tumor cells may form a novel approach in the treatment of cancer.

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