Chapter-II

Literature Review
2.1 Pesticide-induced carcinogenic and genotoxic effects

Pesticides have indubitably benefited human health sector by increasing agricultural yield by controlling pests and also by protecting from insect-borne diseases viz. malaria, dengue, encephalitis, filariasis, etc. But these have some grave drawbacks also, such as potential toxicities to humans and other non target animals. As per Stockholm Convention on Persistent Organic Pollutants, as many as 10 out of the 12 most hazardous and persistent organic chemicals are pesticides (Gilden et al., 2010).

There has been escalation in cancer incidence over the last 50–60 years. Since 1950s is called the “pesticide era” (Murphy, 2005), pesticide use can be very much speculated as one of the reasons if not solely for this increase in cancer incidence. According to WHO (2009), about 300,000 people die from pesticide poisoning each year, with 99% of them being from low- and middle- income countries. Around 600,000 cases and 60,000 deaths occur in India annually, with the most susceptible groups comprising of children, women, workers in the informal sector, and poor farmers (WHO, 2009).

Strong evidences are available for adverse effects arising because of pesticide exposure including neurological, birth defects, fetal deaths (Sanborn et al., 2007), cancers (Bassil et al., 2007) and neurodevelopmental disorders (Jurewicz et al., 2008). Several studies have demonstrated associations between pesticide exposure and various cancers including leukemia, lymphoma, brain, kidney, breast, prostate, pancreas, liver, lung, and skin cancers (McCauley et al., 2006; Bassil et al., 2007; Gilden et al., 2010; Van Maele-Fabry et al., 2010).

In epidemiological studies also, increased risk of cancer has been found associated with both residential and occupational exposures to pesticides (Gilden et al., 2010). Increased incidence of cancer has been observed in farming personnel who apply these pesticides (McCauley et al., 2006). During a study conducted at university of Iowa it was observed that the occupation of golf superintendent greatly amplified one’s odds of getting various cancers such as non-Hodgkin’s lymphoma, brain cancer, lung cancer, large intestine cancer, and prostrate cancer (Kross et al., 1996). Another research project where incidence of brain cancer among 600 people was studied showed that the likelihood of having brain cancer in people who resided within 2600 feet of agricultural vicinity was
enhanced by twofold (Aschengrau et al., 1996). A hospital-based case-control study conducted in France between 2000 and 2004 has showed that occupational exposures to several pesticides were significantly associated with multiple myeloma, Hodgkin’s lymphoma and hairy-cell leukaemia (Orsi et al., 2009). A systematic review in 2007 found that most studies on non-Hodgkin’s lymphoma and leukemia were reporting positive links with pesticides’ exposure and hence recommended that use of pesticides must be reduced (Bassil et al., 2007). A mother's occupational exposure to pesticides during pregnancy can also increase her child's risk of getting leukemia, Wilms' tumor, and brain cancer (Gilden et al., 2010; Van Maele-Fabry et al., 2010). Table 2.1. shows some of the pesticides implicated in various cancers.

Table 2.1. Pesticides suspected of playing a role in certain human cancers resulting from either occupational exposures or non-occupational (environmental) exposures.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Cancer Type</th>
<th>Pesticides</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Hodgkin’s Disease</td>
<td>DDT</td>
</tr>
<tr>
<td>3.</td>
<td>Multiple Myeloma</td>
<td>Dieldrin, Chlorothalonil, Carbon tetrachloride, DDT</td>
</tr>
<tr>
<td>4.</td>
<td>Lung cancer</td>
<td>Dieldrin, Chlorpyrifos, Diazinon, Metolachlor</td>
</tr>
<tr>
<td>5.</td>
<td>Pancreatic cancer</td>
<td>Pendimethalin, 1,3-Dichloropropene, DDT</td>
</tr>
<tr>
<td>6.</td>
<td>Colorectal cancer</td>
<td>Chlorpyrifos, Aldicarb, Chlordane, Dicamba</td>
</tr>
<tr>
<td>7.</td>
<td>Liver cancer</td>
<td>DDT</td>
</tr>
<tr>
<td>8.</td>
<td>Stomach cancer</td>
<td>2,4-D, chlordane, Propargite, Atrazine</td>
</tr>
<tr>
<td>9.</td>
<td>Leukaemia</td>
<td>Mancozeb, DDT, Chlordane/heptachlor, Alachlor</td>
</tr>
<tr>
<td>10.</td>
<td>Brain + CNS (central nervous system)</td>
<td>Pesticides found to increase rates of brain tumours in young and middle-aged horticulturalists</td>
</tr>
<tr>
<td>11.</td>
<td>Bladder cancer</td>
<td>Imazethapyr</td>
</tr>
<tr>
<td>12.</td>
<td>Ovarian cancer</td>
<td>Some evidence to suggest increased risk in female pesticide sprayers</td>
</tr>
<tr>
<td>13.</td>
<td>Breast cancer</td>
<td>Aldrin, Lindane, DDT, Dieldrin, Malathion, Aldicarb</td>
</tr>
</tbody>
</table>
2.2 Mancozeb

Mancozeb \([1,2\text{-ethanediylbis(carbamodithioate)}(2\text{-})]\text{manganese mixture with }[1,2\text{-ethanediylbis(carbamodithioate)}(2\text{-})]\text{zinc}\) (Figure 2.1.) belongs to the ethylene bisdithiocarbamates (EBDCs) group of fungicides. Mancozeb’s close relations: maneb, metiram, propineb, and zineb are the other members of this group.

![Figure 2.1. Chemical Structure of Mancozeb (USEPA, 2005)](image)

2.2.1. Uses

Mancozeb was first marketed in 1944 as a broad spectrum fungicide employed in agriculture, professional turf management, and horticulture. Because of its efficacy against a broad spectrum of fungi and their associated plant diseases, (Belpoggi et al., 2002) its use has increased steadily and today it is one of the most widely used fungicide. Besides agriculture, it is also used in industry as a slimicide in watercooling systems; in sugar, pulp, and paper manufacturing; as a vulcanization accelerator and antioxidant in the rubber industry; and as a scavenger in wastewater treatment because of its chelating properties (Belpoggi et al., 2002).

2.2.2. Biochemical mode of action

Mancozeb *per se* is not fungicidal but can be considered a profungicide which, on getting exposed to water, disintegrates to release ethylene biscisothiocyanate sulfide (EBIS), which subsequently gets transformed into ethylene biscisothiocyanate (EBI) through the action of UV light. Both these products EBIS and EBI are thought to be carrying out active fungicidal activity by inhibiting enzymes containing sulphydryl groups. This lethal
interference with vital enzymatic processes is postulated to obstruct at least six diverse biochemical processes inside the fungal cell cytoplasm and mitochondria (Ludwig and Thorn, 1960; Kaars Sijpesteijn, 1984), thereby resulting in inhibition of spore germination (Szkolnik, 1981; Wicks and Lee, 1982; Wong and Wilcox, 2001).

2.2.3. Spectrum of biological activity

Fungicide Resistance Action Committee has classified mancozeb in mode-of-action group M (Multi Site Action) because it has shown efficacy against a broad range of fungi counting ascomycetes, oomycetes, basidiomycetes, and imperfect fungi. Broad spectrum of activity of this compound endows it efficacy as a fungicide in more than 70 crops and 400 varied diseases, thus contributing to its commercial success (Leader et al., 2008). It is a widely used fungicide in agriculture, professional turf management, and horticulture. In agriculture sector it is used on an extensive range of food/feed crops, counting tree fruits, vegetable crops, field crops, and grapes, ornamental plants, and sod farms. Few other uses comprise greenhouse grown flowers and ornamentals, and seed and seed piece treatment.

2.2.4. Extent of usage

Mancozeb exhibits the traits of a classic multi-site protectant-only fungicide, as when applied onto the target plant, it remains only on the leaf surface rather than penetrating inside where systemic redistribution can take place (Kaars Sijpesteijn, 1982). So as to afford effectual control, a persistent fence made of mancozeb is needed to be present on the leaf surface. Thus, due to being a typical multi-site protectant-only fungicide its use rates and application frequency are needed to be relatively very high so as to compensate the effects of weathering and plant growth, which can rapidly deflate the shield erected by the compound.

As abovementioned it is also ought to be present on the leaf prior to the incursion of fungal spores, and hence it is used in a typical prophylactic manner where applications are made routinely on a calendar spray timetable. However, this approach does not conform to the fundamental doctrines of Integrated Pest Management, where pesticides should not be applied until absolutely indispensable. Furthermore, since it is a broad spectrum multi-site acting pesticide, hence is considered backbone of resistance management programme. Due to these aforementioned grounds, very high quantities of this fungicide are used worldwide. According to USEPA approximately 5.6 million pounds of mancozeb are used annually (USEPA, 2005b). Its greatest use is on potatoes where its application is almost exclusively impelled by the necessity to manage Phytophthora
*infestans* (Gullino et al., 2010), one of the most notorious and devastating organisms in recent human history, being the culprit for the terrible Irish potato (*Solanum tuberosum*) famine in the 1840s which led to death of over 1 million people (Alexopoulos, 1996). Besides potatoes, mancozeb is also used extensively on apples, grapes, onions, pears, tomatoes, squash, and melons (Gullino et al., 2010).

### 2.2.5. Carcinogenic potential

Animal bioassays have revealed its tumorigenic potential in many organs (pancreas, thyroid, liver, breast and skin). Mancozeb was found to promote pancreatic tumors in rats fed a diet containing 100 mg/kg b wt. mancozeb for 24 weeks (Monis and Valentich, 1993). USEPA (2005a) demonstrates that the thyroid gland is the target organ for Mancozeb and thyroid toxicity has been manifested as alterations in thyroid hormones, increased thyroid weight, and microscopic thyroid lesions, and thyroid tumors. Mancozeb is a developmental and reproductive toxin (Kegley et al., 2011). Mancozeb or its metabolites are capable of causing transplacental carcinogenesis in mice by crossing the placental barrier, exerting DNA damage and tumor initiating consequences in the fetal cells that, after promotion with 12-O-tetradecanoyl-phorbol-13-acetate (TPA), get converted into neoplastic cells (Shukla and Arora, 2001). Furthermore, in two studies on rats it was shown that high intake of mancozeb affected the estrous cycle (Mahadevaswami et al., 1999; Baligar and Kaliwar, 2001). Cecconi et al. (2007) suggest with regard to both *in vitro* studies on mouse embryos and a reproductive *in vivo* study on mice (Rossi et al., 2006), that mancozeb has an effect on female infertility and that it also can induce ovarian cancer. In a study by Belpoggi et al. (2002) mancozeb was found to act as a multipotent carcinogen inducing a variety of tumors of different origin in rats fed a diet containing 10–1000 ppm for 104 weeks. Our lab has pioneered in establishing its carcinogenic potential in skin. Mancozeb topically applied at a dosage of 100 mg/kg body weight 3 times per week for 7 months was found to promote skin tumors in mice (Shukla et al., 1990).

Evidence for effects in humans is currently less well founded (Lindh et al., 2008). Concerning occupational exposure to pesticides it can also be difficult to differentiate what substance that leads to a specific effect since workers normally are exposed to a vast quantity of different compounds in different combinations (Leiphon and Picklo, 2006). Besides, little is known about additive, protective or synergic effects of combinations of pesticides. Thus, hitherto, epidemiology studies could not help researchers to accurately ascertain which pesticides are linked to which cancers.
However some epidemiology studies have also associated mancozeb to birth defects and cancers like lymphphaematopoeitic, skin and thyroid cancer (Mills et al., 2005; Dennis et al., 2010). The study on banana plantation workers in the Philippines indicated that high exposure to mancozeb correlated with enlarged thyroid glands (Panganiban et al., 2004). Steenland et al. (1997) found an increase in thyroid stimulating hormone among Mexican workers exposed to EBDCs which can have adverse effects on the thyroid glands. They also found increases in sister chromatoid exchanges as well as in frequency of chromosome aberrations, which also is demonstrated in another study conducted by Jablonická et al. (1989).

2.2.6. Skin: major route for mancozeb exposure

Skin is the major route of exposure to mancozeb (USEPA, 1987), thus this research work has been conducted to ascertain mancozeb’s carcinogenic potential in skin. Mancozeb is a coordination product of Zn and another EBDC pesticide, Maneb that has apparently been responsible for some cases of chronic skin diseases in occupationally exposed workers (Fishel, 2008). Mancozeb itself is also reported to cause skin sensitization, chronic skin disease in exposed workers (USEPA, 1999). In animals its skin carcinogenic potential has been demonstrated in several studies (Mehrotra et al., 1987; Shukla et al., 1990; Gupta and Mehrotra, 1992), but this information in humans is lacking, thus studies in human system are warranted.

2.2.7. Regulatory status

Due to high risks of adverse effects on human health, USEPA in 1989 cancelled most of the uses of mancozeb. But, in 1992 it reinstated some of the proposed uses which were intended to be cancelled, despite acknowledging that exposure to EBDCs may pose increased occupational health risks of cancer, birth defects and thyroid disorders. In 1992 USEPA also concluded that dietary risks of EBDCs exceeded the benefits for the uses for which they were registered (EBDC, 1998).

In 1997 Food and Agriculture Organisation (FAO)/WHO International Codex Committee Joint Meeting on Pesticides Residues declared that EBDCs were toxic to thyroid (WHO, 1997). There are studies confirming mancozeb’s endocrine disruptor potential and this made European Union (EU) to place mancozeb in its category 1 endocrine disruptors list. It is on PAN Bad Actors chemicals list, as it has many negative characteristics: potentionally ground water contaminator, suspected endocrine disruptor, developmental or reproductive toxic (Kegley et al., 2011). In the USA, EPA included mancozeb on its Hazardous Air Pollutants list (Kegley et al., 2011). In Sweden, there were concerns
about EBDCs since 1990s. Mancozeb was classified as carcinogenic and severely restricted there. In Norway, it was phased out by 2000. But in India mancozeb is registered for use under section 9(3) of the insecticides act, 1968 and has unrestricted accessibility.

Although mancozeb has been commercially produced for almost 60 years, information regarding its carcinogenic potential in humans is still inadequate, due to this inadequacy; various agencies have very contradictory and confusing stands on mancozeb’s carcinogenic risk (Table 2.2.). Inspite of being listed on the most hazardous pesticide list by PAN (PAN HHP, 2009) and being referred to as class B2 probable human carcinogen by USEPA (USEPA, 2005a), mancozeb is still very much in use with an annual consumption of as much as ~5.6 million pounds (USEPA, 2005b). Thus, there is an urgent need for studies evaluating the mancozeb’s neoplastic potential in human cells.

**Table 2.2.** Carcinogenic potential of mancozeb shown by various regulatory authorities and advisory bodies.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Regulatory Agency</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>IARC</td>
<td>Not Listed</td>
</tr>
<tr>
<td>2.</td>
<td>U.S. NTP</td>
<td>Not Listed</td>
</tr>
<tr>
<td>3.</td>
<td>California’s Proposition 65 Known Carcinogens</td>
<td>Yes</td>
</tr>
<tr>
<td>5.</td>
<td>TRI</td>
<td>Yes</td>
</tr>
</tbody>
</table>

(Kegley et al., 2011)


**2.3. Carcinogenicity assessment of pesticides**

Evidence for identifying cancer-causing pesticides comes from three types of studies (NCI, 2004), each of them plays key role in enabling public health officials to formulate regulatory decisions about pesticide use:

- Human epidemiological studies
• In vivo animal studies
• In vitro cell culture studies

2.3.1. Human epidemiological studies

The most certain mode is to observe whether they have induced cancer in human population where epidemiologists design studies that follow certain populations over time to monitor whether a specific substance (e.g., arsenic or benzene) or exposure (e.g., sunlight or smoking) is likely to induce cancer (NCI, 2004). They also compare the exposure histories of individuals who have developed cancer to those who have not developed cancer at a particular point in time. But human epidemiology studies that have been done so far share common problems. First, they usually involve a small number of people which makes it highly probable that their results are down to chance. Second, it is very difficult to find out the total pesticide amounts that people were actually exposed to. Third, there is a large variety of pesticides and it is not easy to ascertain which of those can lead to the risk of cancer. Fourth, the results from different studies lack consistency (NCI, 2004).

2.3.2. In vivo animal studies

So far, human studies could not allow researchers to accurately ascertain which pesticides are linked to which cancers and hence, the need arises for other testing methods involving laboratory experimentation. In fact, a very important advantage of these tests over epidemiology studies is that they allow researchers to anticipate potential carcinogenic exposures before they cause large numbers of human cancers. In experiments where animals are used, mice or rats are most commonly utilized to investigate for cancer-causing substances because they are smaller, easier to handle, and more economical than larger animals. In addition, they are quite analogous to humans in their response to carcinogens and thus, most major forms of human cancer have been reproduced in these animals through exposure to chemical carcinogens. Since the lifetime of rodents is short viz. only two to three years, they make the information about the cancer-causing potential of test substances available relatively quickly. Many special strains of rodents which are particularly suitable for cancer testing have also been developed (NCI, 2004).

But this usage of animals is a complicated issue. For example, though animal models (e.g. monkeys and baboons) are considered most valuable for the extrapolation of the results to humans, ethical reasons compel to shun their usage. In addition, due to
differences among different species, the results obtained in animal models will never be perfectly relevant to humans. Then, there is a strong animal welfare case against such animal experimentation. Nonetheless, an equally compelling argument can be made that it would be inappropriate to deny the benefits of animal experimentation until apt alternatives to animal procedures can be established (NCI, 2004).

2.3.3. In vitro studies

To resolve above issues, European Centre for the Validation of Alternative Methods (ECVAM) was created in 1991, with its central goal, as defined by its Scientific Advisory Committee in 1993, is to promote the scientific and regulatory acceptance of alternative approaches which are of significance to the biosciences and which reduce, refine or replace the use of laboratory animals (Bottini et al., 2008). As part of this continuing endeavor to reduce the use of animals in testing for cancer, researchers are using cells grown in the laboratory. Here, cells are exposed to potential carcinogens and are then monitored to see whether molecular features characteristic of cancer cells develop. Besides reducing the animal usage, this type of studies have other advantages also like these can be carried out more quickly and economically and can be helpful in assessing whether to perform further studies in animals. These results from laboratory experiments also give clues to epidemiologists regarding which hypotheses to investigate in human population studies (NCI, 2004).

2.3.4. Carcinogenesis and cell transformation

Carcinogenesis is a multistep process, which involves sequential genetic alterations in a single target cell, which cause stable alterations in growth control and culminate in cells that are able to form malignant tumors (Barrett, 1993; Maronpot, 1999). Investigations appraising the carcinogenic risks of various compounds and explicating the underlying mechanisms of carcinogenesis have entailed the use of experimental animal models, in vitro cellular systems as well as clinical and epidemiological studies. The traditional practice for these explorations is the lifetime rodent bioassays with pathological examination of tissues. These assays are employed to identify complete carcinogens, tumor promoters as well as co-carcinogens. But there are some disadvantages for instance these rodent bioassays are time consuming, labor-intensive and costly. Moreover, due to species differences, data from rodents does not correlate well with humans, and thus extrapolation of the information to humans is complicated and problematic (Combes, 1997; Gottmann et al., 2001). Thus, the discovery of in vitro morphological transformation of mammalian cells in culture some 35 years ago became
a boon for the carcinogenesis studies (Berwald and Sachs, 1965; Meyer, 1983). The phenomenon of morphological cellular transformation entails modifications in the behavior and growth control of cells, portrayed by one or more characteristics like alterations in cellular morphology, disordered pattern of colony growth; and acquisition of anchorage independent growth property that are the traits of tumorigenic cells (Figure 2.2.) (Barrett and Fletcher, 1987; Yuspa and Poirier, 1988). Basically, the chief endpoints of cell transformation viz. focus formation and the growth capacity in soft-agar emerge as a result of loss of contact inhibition and of anchorage dependence, respectively. These phenotypic modifications arise from alterations in the expression of tumor suppressor genes and/or oncogenes, and, can be elicited by exposing normal cells to carcinogens, or by expressing activated oncogenes in those normal cells. These transformed cells which have attained all the traits of malignant cells achieve the competence to form invasive tumors in vulnerable animals (Barrett et al., 1986).

![Figure 2.2.](image_url) Cancer-associated cellular traits that are progressively adopted by normal cells when becoming cancerous. (Hanahan and Weinberg, 2000)
2.3.5. Human cell based transformation systems

Though the mechanisms accountable for rodent and human carcinogenicity have not completely comprehended yet, it is believed that the progression of the transformation of rodent and human cells is analogous or virtually alike (Fusenig and Boukamp, 1994). Nevertheless, there are many disparities between rodents and humans due to difference in species, which are critical with reference to cancer development. Some of them are: a) rodents live for only 2-3 years, whereas, humans have a relatively longer lifespan with cancer occurring with higher frequency in late stages of lifetime, b) dissimilarities in xenobiotics’ metabolism; c) relatively higher frequencies of both spontaneous and induced immortalizations and transformations in cultured rodent cells in comparison to human cells.

Thus, to assess the human carcinogenic risk associated with various pesticides an ideal transformation assay would employ human cells. Hitherto, it has been difficult to develop human cell transformation systems for this purpose with tumorigenicity being the final endpoint. The major difficulty is that in contrast to animal cells in culture, cultured human cells do not spontaneously get immortalized.

Since the sequential selection of cells with apposite mutations in oncogenes and/or tumor suppressor genes is involved during the course carcinogenic progression, nonimmortalized cells senescence before acquiring all the genetic alterations crucial for tumorigenicity. Due to this reason, the existing human cell transformation system utilizes genetically altered cell lines, rather than primary cultures, which have attained an immortalized phenotype (Mc Cormick and Maher, 1989; Fusenig and Boukamp, 1994).

2.3.6. HaCaT: in vitro model system for human skin keratinocyte carcinogenesis

HaCaT cell line was derived from spontaneous immortalization of normal human keratinocytes, most likely caused by mutations in the p53 gene, and the ensuing loss of genes involved in senescence. HaCaT cell line has been established as an in vitro model system for human skin keratinocyte carcinogenesis (Boukamp et al., 1988; 1997). The name, HaCaT, denotes that it had originated from normal human adult skin keratinocytes through prolonged cultivation at a diminished Ca²⁺ concentration and elevated temperature. This spontaneously immortalized cell line (>140 passages) retains a stable non-tumorigenic phenotype and also shows good predictive results in comparison with other keratinocyte and skin models. Thus, they have become a widely used in vitro model, a paradigm for normal skin keratinocytes (Fusenig and Boukamp, 1994; 1998).
For studies appraising the tumor progression by various carcinogenic agents this immortalized cell line is an important model (Fusenig and Boukamp, 1998). Actually, both at genotypic and phenotypic levels this model fits quite well the different stages of human epithelial skin carcinomas in situ as well as of derived skin carcinoma cell lines and this allows the identification of genetic alterations and phenotypic characterizations coupled with various stages of transformation.

2.4. Understanding the mechanism of carcinogenesis induced by various carcinogens

Studies deciphering the underlying mechanism of mancozeb-induced damage can be very instrumental in finding appropriate antidotes for mancozeb’s toxicity.

On the basis of causal mechanisms, carcinogens can be broadly divided into several classes (Table 2.3).

(1) **Genotoxic carcinogens**, if they react with nucleic acids. These can be directly acting or primary carcinogens, if they are of such reactivity so as to directly affect cellular constituents.

(2) Alternatively, they may be **procarcinogens** that require metabolic activation to induce carcinogenesis.

(3) **Epigenetic carcinogens** are those that are not genotoxic.

Multiple mechanisms of carcinogenesis adopted by various carcinogens has been depicted in (Figure 2.3.). These potential mutagens/carcinogens exert their genotoxic effects depending on their cellular target(s). If these mutations are induced by carcinogen-DNA adducts, the mutations observed in tumors should correlate with the DNA-binding characteristics of the carcinogen.

Mutagens can also induce genomic changes by targeting DNA directly and/or indirectly, by binding to proteins involved in the maintenance of genome integrity (e.g. tubulins, DNA repair enzymes, proteins involved in the control of cell cycle etc.) (Nohmi et al., 2005). This dialect between the carcinogen and DNA endows the cell with the infidelity for replication rendering it to incorporate errors during the process; hence failing to reproduce into otherwise intended normal cell. This is the initiation of mutation, or mutagenesis, and without proper execution of DNA repair (DNA repair happens naturally under normal circumstances), the cell propensities towards carcinogenesis.
### Table 2.3. Types of carcinogen

<table>
<thead>
<tr>
<th>Type</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Genotoxic carcinogen</strong></td>
<td><strong>Example</strong></td>
</tr>
<tr>
<td>Primary, direct-acting alkylating agents</td>
<td>Dimethylsulfate, ethylene imine, b-propiolactonel, Ethyl methanesulphonate</td>
</tr>
<tr>
<td><strong>2. Procarcinogens</strong></td>
<td>7,12-dimethylbenz(a)anthracene, Benzo[a]pyrene</td>
</tr>
<tr>
<td>Polycyclic aromatic hydrocarbons</td>
<td></td>
</tr>
<tr>
<td>Nitrosamines</td>
<td>Dimethylnitrosamine</td>
</tr>
<tr>
<td>Hydrazine</td>
<td>1,2-Dimethylhydrazine</td>
</tr>
<tr>
<td>Inorganic</td>
<td>Cadmium, plutonium</td>
</tr>
<tr>
<td><strong>3. Epigenetic carcinogens</strong></td>
<td><strong>Example</strong></td>
</tr>
<tr>
<td>Promoters</td>
<td>Phorbol esters, saccharin, bile acids</td>
</tr>
<tr>
<td>Solid state</td>
<td>Asbestos, plastic</td>
</tr>
<tr>
<td>Hormones</td>
<td>Estrogens</td>
</tr>
<tr>
<td>Immunosuppressants</td>
<td>Purine analogues</td>
</tr>
<tr>
<td>Cocarcinogens</td>
<td>Catechol</td>
</tr>
<tr>
<td><strong>4. Unclassified</strong></td>
<td><strong>Example</strong></td>
</tr>
<tr>
<td>Peroxisome proliferators</td>
<td>Clofibrate, phthalate esters</td>
</tr>
</tbody>
</table>

(Adapted from Woo and Lai, 2003)
Mutagens can induce genotoxic effects and cancer either by targeting DNA directly or indirectly, by binding to proteins involved in the maintenance of genome integrity (e.g. tubulins, DNA repair enzymes, proteins involved in the control of the cell cycle etc.). Non-genotoxic compounds are also capable to induce cancer by increasing cell proliferation rate (e.g. mitogens), by changing the DNA methylation status or by triggering cytotoxicity. Cell proliferation can be a primary effect of the carcinogen or a secondary effect consequent to cell toxicity. Apoptosis can be induced by several types of genotoxicants. Excessive elimination of cells by apoptosis can induce compensatory cell proliferation to restore homeostasis. Carcinogenesis is therefore the result of the balance between mutations, epigenetic changes, cell proliferation and cell death.
2.4.1. RNAi in unraveling the causal mechanisms of pesticide-toxicity

Mechanistic understanding of the consequences of chemicals on biological systems can aid in rational risk appraisals. RNAi mechanism has the potential for unraveling the pathogenesis and mode of action of pesticides. In a study on 2,2',4,6,6'-pentachlorobiphenyl (PCB 104) exposed human microvascular endothelial cells Janus kinase 3 (JAK3) siRNA and Epidermal Growth Factor Receptor (EGFR) siRNA have been used to identify the role of EGFR, JAK3 and the Mitogen Activated Protein Kinase (MAPK) signaling pathways in PCB-induced up-regulation of Matrix Metalloproteinase(MMP)-3 leading to acceleration of transendothelial migration of tumor cells (Eum et al., 2006).

Several studies have indicated that exposure to pesticides can increase the risk of parkinson’s disease (PD) (Gatto et al., 2010). In a recent epidemiological study two pesticides rotenone and paraquat have shown link with PD. People who used either of the two pesticides have 2.5 times higher risk of developing Parkinson’s disease (Tanner et al., 2011). But the underlying mechanism is not known. RNAi is being applied extensively to elucidate this mechanism.

1-Methyl-4-phenylpyridinium [MPP(+)]] is a neurotoxin reported to cause PD in experimental animals and humans (Kalivendi et al., 2004). The chloride of MPP+ is used as a herbicide under the trade name cyperquat and is structurally similar to the herbicide paraquat. In the pathogenesis of PD, neurodegeneration is mainly constrained to dopaminergic neurons that have cytoplasmic inclusions called Lewy bodies. These bodies contain the protein alpha-synuclein and from recent studies it is evident that levels of alpha-synuclein expression are a major factor contributing in PD pathogenesis. Alpha-synuclein overexpression has been found to be positively correlated with the severity of disease in PD patients. However, data regarding the effects of suppressing alpha-synuclein expression in human neurons was scarce. By employing the technique of RNAi, Fountaine and Wade-Martins (2007) could manage to show for the first time that knockdown of alpha-synuclein protects human dopaminergic neuroblastoma cells from MPP(+) toxicity and reduces dopamine transport. Yang et al. (2004) also have used RNAi to demonstrate that caspase-3-dependent proteolytic activation of protein kinase Cα contributes to the neurotoxin MPP+ induced degenerative process in dopaminergic neurons.

Neuron-restrictive silencer factor (NRSF)/neuronal repressor element-1 silencing transcription factor (REST) and its neuron-specific truncated form REST4 are involved in
pathogenesis of various nervous system diseases, such as global ischemia, epilepsy and Huntington disease. Yu et al. (2009) investigated the alterations of NRSF and REST4 in a cellular model of PD. Neurotoxin MPP+ was observed to induce the mRNA and protein expression of NRSF and REST4 in SH-SY5Y cells. Further validation of the involvement of NRSF and REST4 in MPP+ induced deleterious effects in SH-SY5Y cells was carried out by employing RNAi technique (Yu et al., 2009).

Rotenone, a broad spectrum pesticide, is an inhibitor of mitochondrial complex I and induces apoptosis in cells. However, little is known about the underlying mechanism. Tsuruta et al. (2007) used RNAi to determine the role of caspase-activated DNase (CAD) in rotenone-induced apoptosis. Results suggested that CAD is the endonuclease mediating internucleosomal DNA fragmentation in rotenone-induced apoptosis (Tsuruta et al., 2007). Mutations in leucine-rich repeat kinase 2 (LRRK2) are prevalent causes of late-onset PD but the underlying mechanism is not known. In a study where RNAi knockdown of the endogenous orthologs for p38, sek-1 and pmk-1 in rotenone-exposed C. elegans, was carried out, LRRK2-mediated protection against mitochondrial stress was found to be abolished. This study helped in understanding that LRRK2 requires sek-1 and pmk-1 for its action (Hsu et al., 2010). These reports assert that although data accumulated on the applications of RNAi in deciphering the mechanism of pesticide-induced toxicity/carcinogenecity is scarce, yet this technique carries excellent potential in this regard.

2.4.2. Role of oxidative stress in chemical-induced carcinogenesis

Recent studies have established a vital role for oxidative stress in tumorigenesis (Ishikawa et al., 2008; Kumar et al., 2008). Oxidative stress arises due to an imbalance between the generation of reactive oxygen and the ability of the biological system to detoxify the reactive intermediates or repair the ensuing oxidative damage. Thus, oxidative stress is ascribed as a critical pathophysiological mechanism in various pathologies, including cardiovascular diseases, cancer, diabetes, rheumatoid arthritis, or neurological disorders like Alzheimer or Parkinson disease (Dhalla et al., 2000; Sayre et al., 2001; Jenner, 2003; Dalle-Donne et al., 2006; Valko et al., 2007). In case of cancer reactive oxygen species (ROS) has been shown to incite all the three stages of cancer namely, initiation, promotion and progression. ROS can be generated from both endogenous sources like mitochondria, peroxisomes, and inflammatory cell activation (Klaunig and Kamendulis, 2004) as well as from exogenous sources, such as environmental agents, pharmaceuticals, and industrial chemicals. Figure 2.4 shows the prospective consequences of reactive oxidative species when not offset by antioxidant defenses of the cell.
This oxidative stress then, in turn, may cause DNA, protein, and/or lipid damage, leading to changes in chromosome instability, genetic mutation, and/or modulation of cell growth.
that may result in cancer. ROS can also directly induce the expression of a variety of transcriptional factors involved in neoplastic transformation (Toledano and Leonard, 1991; Wei et al., 1992). Thus, chemical and physical agents including those that induce reactive oxygen species can induce and/or modulate this multistep process.

2.4.3. Oxidative damage caused by pesticides

Literature reports assert that the major mechanism underlying pesticides' toxicity is via alterations in the cellular oxidative status. Pesticides can induce oxidative stress via a multi-step pathway, resulting in an imbalance between pro-oxidant and antioxidant defense mechanisms in different tissues, including alterations in antioxidant enzymes (Banerjee et al., 2001). Endosulfan (13 mg/kg/day), an organochlorine insecticide, caused degeneration in mouse kidney due to oxidative stress (Caglar et al., 2003) Exposure to the insecticide, rotenone, increased xanthine oxidase enzyme activity and lipid peroxidation (LPO) in liver tissue (Terzi et al., 2004). The pyrethroid insecticide, cypermethrin, significantly ($p<0.05$) induced free radical production in plasma, liver, brain and testes (El-Demerdash et al., 2003). Levels of malondialdehyde (MDA), a major oxidation product of peroxidized polyunsaturated fatty acids, have been considered as an important indicator of lipid peroxidation (Kalender et al., 2004). Significant dose-dependent depletion of glutathione (GSH) levels and perturbations in antioxidant enzyme levels further confirmed the potential of the insecticide, fenvalerate to induce oxidative stress in hepatic tissue (Prasanthi et al., 2005). Subchronic exposure to the insecticide, dimethoate (6 and 30 mg/kg b wt) resulted in a decrease in GSH levels in both liver and brain tissues of male Wistar rats (Sharma et al., 2005). It was concluded that the oxidative stress due to dimethoate may be ascribed to the induction of Cytochrome P450, inhibition of acetylcholinesterase and disturbance in the activity of glutathione-S-transferase (GST) enzyme causing lipid peroxidation and histological changes in the liver and the brain (Sharma et al., 2005).

Piperonyl butoxide (PBO), an insecticide, has a liver-tumor-promoting effect increasing production of ROS as a byproduct of hepatic microsomal oxidation in mice (Muguruma et al., 2006; Muguruma et al., 2007; Kawai et al., 2010). The increase of ROS is due to PBO-induced regulation of glutathione reductase (GR), NAD(P)H: quinone oxidoreductase 1, and other antioxidant enzymes. This regulation is under the transcriptional factor Nrf2 (Muguruma et al., 2007). PBO is capable of increasing the gene expression of CYP1A1, a cytochrome P-450 isoform and the most active enzyme catalyzing procarcinogens (Muguruma et al., 2006).
Chloropyriphos ethyl, an organophosphorus pesticide, induced oxidative stress by reducing SOD and GSH antioxidant enzymes’ activity, and, thereby resulted in tissue damage in the liver, kidney, brain and fetus in pregnant albino Wistar rats (Zama et al., 2007).

2.4.4. Proxidant activity of mancozeb

While the causal mechanism(s) behind the toxicity of other categories of dithiocarbamate pesticides has been frequently related to the quick breakdown and release of carbon disulfide or ethylene thiourea (ETU), which are main metabolites of dithiocarbamate pesticides, metal–EBDC pesticides like mancozeb are found to be more stable (Engst and Schnaak, 1967; Fishbein, 1976). Thus, there must be some other alternative mechanism to explain Mancozeb’s toxicity. Mancozeb’s ability to act as a prooxidant agent could be the underlying mechanism. Several reports have lately provided support to this hypothesis. Maneb and Zineb, relatives of mancozeb, were recently reported to induce catechol auto-oxidation (Fitsanakis et al., 2002). Treatment with antioxidants ascorbic acid or α-tocopherol was found capable to reverting sperm cell alterations inflicted by mancozeb exposure in rats (Khan and Sinha, 1994; Kackar et al., 1997). Chromatid exchange in Chinese hamster ovary cells induced by Zineb exposure was also inhibited by antioxidants ascorbic acid or α-tocopherol (Soloneski et al., 2003). In mesencephalic cells ROS generated by mancozeb contributed to its neuronal toxicity (Domico et al., 2007). In RAT-1 fibroblasts cultured in vitro and in peripheral blood mononucleated cells (PBMC) isolated from Wistar rats mancozeb-induced genotoxic damage and apoptosis were ascribed to generation of oxidative stress (Calviello et al., 2006).

Researchers are currently suspecting that the presence of metals that are known to catalyze the formation of ROS through the Fenton reaction can be the reason for the observed prooxidant activity of mancozeb (Calviello et al., 2006).

2.5. Role of dietary compounds in preventing pesticide-induced genotoxicity and carcinogenicity

Human body has defense mechanism to withstand low levels of toxic insults and human diet plays a critical role in sustaining the overall health by strengthening these innate defenses of the body. By acting as a key factor in maintaining the genomic stability diet influences all crucial pathways such as exposure to dietary carcinogens, DNA repair, DNA synthesis and apoptosis. The harmful effects of toxicants can be assuaged by plant
products fruits, vegetables encompassing few spices, which furnish the essential antioxidants and other therapeutic molecules.

Fruits and vegetables contain high amounts of cancer fighting compounds such as flavanoids which not only neutralize deleterious free radicals and thus prevent their accretion inside the cells but also modulate cell signaling pathways. This modulation of cell signaling pathways by antioxidants could help prevent cancer by (i) preserving normal cell cycle regulation; (ii) inhibiting proliferation and inducing apoptosis; (iii) inhibiting tumour invasion and angiogenesis; (iv) suppressing inflammation; (v) stimulating phase II detoxification enzyme activity and other effects (Valko et al., 2007). In conjugation pathway which is the second phase of detoxification, liver appends chemical entities such as cysteine, glycine or a sulphur molecule to the toxin thereby making it water soluble for subsequent removal from the body. For efficacious Phase II detoxification, liver cells need sulphur containing amino acids for instance taurine and cysteine along with glycine, glutamine, choline and inositol. Eggs, cruciferous vegetables such as broccoli (sulphoraphane), cabbage, brussels sprouts and cauliflower, raw garlic, onions, leek and shallots are superb suppliers of natural sulphur compounds boosting the Phase II detoxification. Thus, such foods can be construed to possess good cleansing potential. In fact, the acidic solutions (radish, citric acid, ascorbic acid, acetic acid and hydrogen peroxide) are found to be effective in eliminating organochlorine and organophosphorus pesticides from contaminated potatoes (Zohair, 2001).

Vitamin E (α-tocopherol) a potent antioxidant is effective in removing the damage to DNA and eliminating the cytotoxic effect of malaoxon which is a breakdown product of pesticide malathion and is considered about 100 times more toxic than malathion (Blasiak and Dorota Stańkowska, 2001). This protective action of α-tocopherol indicates that it can act as a protective agent against DNA damage in people occupationally exposed to malathion and maloxon.

The phenolic derivatives from several spices have strong anticarcinogenic activities. Curcumin in turmeric (Curcuma longa L., Zingiberaceae), [6]-gingerol in ginger (Zingiber officinale Roscoe, Zingiberaceae) and capsaisin in chilli (Capsicum annuum L. Solanaceae) have effectual anticarcinogenic potential.

These phytochemicals impart chemoprotective effects chiefly through anti-oxidative and anti-inflammatory properties. Bell pepper and black pepper are efficacious in inhibiting the mutational effects inflicted by ethyl carbomate (El Hamss et al., 2003). In another study aqueous black tea extract has presented significant protection against pesticide-induced hepato-oxidative damage (Khan et al., 2005a). Ginger has been found to exert
protective effects against lindane by modulating lindane-induced oxidative stress. Ginger significantly abrogated lindane-induced LPO, simultaneously modulating oxygen free radical scavenging enzymes as well as GSH and the GSH dependent enzymes glutathione peroxidase, GR and GST (Ahmed et al., 2008). Ginger has shown efficacy against malathion also by attenuating malathion-induced LPO and oxidative stress in rats (Ahmed et al., 2000a). Danshensu, a natural phenolic acids isolated from the root of Salvia miltiorrhiza Bunge, has been reported to possess protective potential against hepatic injury induced by omethoate, an organophosphorous insecticide, in rats and the underlying pharmacological mechanism is related to the anti-inflammatory effect of Danshensu (Ren et al., 2010). These reports suggest that a diet containing such naturally occurring pharmacologically active compounds can be very effective in exerting protective effects against damage caused by exposure to environmental agents like pesticides by ameliorating oxidative stress and replenishing of the antioxidant status. [6]-Gingerol, one of the major constituents of ginger, has very potent antioxidant and anti-inflammatory properties. There are many studies reporting [6]-gingerol’s good chemopreventive properties, thus [6]-gingerol has been explored in this research work for its preventive potential against mancozeb-induced genotoxic and neoplastic effects.

2.5.1. Ginger and its pharmacological constituents

Ginger, a brown color, tropical root native to Southeast Asia, has been used as a spice and as a medicine for centuries. Ginger takes its name from a Sanskrit word stringa-vera which means ‘horn like body’, because it resembles the antlers of a deer. In 1807 an English botanist William Roscoe (1753-1831) bestowed the plant with the name Zingiber officinale (family: Zingiberaceae). Due to its important pharmacological properties it is mentioned in the writings of Confucius and has been important in Chinese medicine for many centuries. Its mention in the Koran, the sacred book of the Muslims, shows it was recognized in Arab countries as far back as 650 A.D. In Western Europe it was one of the earliest spices known, used since the ninth century. It was so much liked in Europe that it got incorporated in every table setting, just as salt and pepper. Some of the other names of this spice are: East Indian Pepper, Jamaica Ginger, Jamaica Pepper; Gingembre (French); Ingwer (German); Zenzero (Italian); Jengibre (Spanish); Cheung, Chiang, Jeung (Burmese); Adruk (green), Ard(r)Ak(h) (green), Sont(h) (dried) (Indian); Aliah (Indonesian); Mioga, Myoga, Shoga (Japanese); K(h)ing (green) (Thai) (Coates, 2005).

Ginger is very widely utilized in Ayurvedic and Western herbal medicines to treat arthritis, rheumatic disorders and muscular discomfort and in traditional Chinese medical practice.
for the treatment of headaches, nausea, febrile conditions and colds (Dedov et al., 2002; Grzanna et al., 2005). In South Africa, the fresh or dried ginger rhizomes are used as stomachics and tonics for treating dyspepsia, flatulence and nausea (Van Wyk et al., 2002). In other parts of the world also the dried ginger rhizomes are used for various human ailments, including the treatment, management and/or control of diarrhoea, dysentery, ulcers, boils, wounds, fever and cough. Some of the other recognized pharmacological activities of ginger rhizomes are antimicrobial, analgesic, antipyretic, antiemetic, antiulcer, anxiolytic, cardiotonic, antihypertensive, hypoglycaemic, antihyperlipidaemic, antiinflammatory and immuno-stimulant properties (Gupta and Sharma, 2001; Ghayur and Gilani, 2005; Grzanna et al., 2005) (Figure 2.5). Besides these well documented effects of ginger and its constituents, few studies have also revealed that some of the constituents of ginger have potential to play a key role in the prevention of various human cancers (Shukla and Singh, 2007).

There are numerous constituents of ginger whose composition in rhizomes varies depending on the place of origin and also on whether the rhizomes are fresh or dry. The pungency of fresh ginger is primarily because of the gingerols, which are a homologous series of phenolic ketones while the sensory perception of ginger in the mouth and the nose are due to two different groups of chemicals namely Volatile Oils and Non-Volatile Pungent Compounds. Some of the important volatile oil components in ginger are sesquiterpene hydrocarbons, predominantly zingerene (35%), curcumene (18%) and farnesene (10%), with lesser amounts of bisabolene and b-sesquiphellandrene. There is a small fraction of approx. 40 different monoterpenoid hydrocarbons with 1, 8-cineole, linalool, borneol, neral, and geraniol being the most copious (Shukla and Singh, 2007 and refs. cited therein). Non-volatile pungent principles such as the gingerols, shogaols, paradols and zingerone are the biologically active constituents of ginger rhizome. The gingerols, a series of chemical homologs varying in the length of the unbranched alkyl chains, were recognized as the main active constituents in the fresh rhizome (Shukla and Singh, 2007 and refs. cited therein). [6]-Gingerol is the major gingerol while [8]- and [10]-gingerol are present in lesser amounts. The gingerols being thermally unstable get transformed under elevated temperatures to [6]-, [8]-, and [10]-shogaol (after shoga, the Japanese term for ginger), another homologous series and the dehydrated form of the gingerols. These shogaols on hydrogenation transform to another form Paradols which are similar to the gingerols. Besides the extractable oleoresins, ginger also contains many carbohydrates, fats, waxes, vitamins and minerals. Ginger rhizomes have a strong proteolytic enzyme called zingibain.
2.5.2. Antioxidant and anti-inflammatory effects of ginger and [6]-gingerol

In view of the fact that tumor promotion is closely associated to inflammation and oxidative stress, an agent that displays anti-inflammatory and/or antioxidant properties could play a key role as an anticarcinogenic agent. Ginger has substantial antioxidant and anti-inflammatory potency. Due to these properties ginger demonstrated protection against Malathion (a pesticide)-induced oxidative stress in rats where it was introduced via the rats' diets (Ahmed et al., 2000a). The antioxidant efficacy of ginger was revealed
to be as potent as vitamin C in reducing lipid peroxidation in rats by manipulating the enzymatic blood levels of antioxidant enzymes viz. superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (Ahmed et al., 2000b). A significant positive influence of ginger on plasma lipid composition that may be responsible for the impediment of atherosclerotic processes was also shown in apolipoprotein E-deficient mice (i.e., mice that are prone to develop atherosclerosis). A significant reduction in the basal concentration of low-density lipoprotein-associated lipid peroxides was seen in mice that consumed ginger in their drinking water (Fuhrman et al., 2000).

[6]-Gingerol which is one of the major constituents of ginger also has very potent antioxidant and anti-inflammatory properties. This has evidently been shown by suppression of phospholipid peroxidation induced by the \( \text{FeCl}_3 \)-ascorbate system and inhibition of xanthine oxidase system which is responsible for the generation of reactive oxygen species, such as superoxide anion on [6]-gingerol treatment (Aeschbach et al., 1994). [6]-Gingerol also inhibited the arachidonic acid induced platelet aggregation, and, formation of thromboxane B2 and prostaglandin D2 in a concentration-dependent (0.5–10 \( \mu \)M) manner (Shukla and Singh, 2007 and refs. cited therein).

[6]-Gingerol is also endowed with many other pharmacological properties like antimicrobial, analgesic, hypoglycaemic (Langner et al., 1998; Coates, 2005) and cardioprotective (Ghayur et al., 2005; Lam et al., 2007).

2.5.3. Cancer chemopreventive effects of ginger and [6]-gingerol

The rhizome of ginger has been demonstrated to have antitumor promotional potential as manifested by attenuation of 12-O-hexadecanoylphorbol-13-acetate (HPA), a phorbol-ester promoter, incited Epstein–Barr virus activation in Raji cells. Treatment of Jurkat human T cell leukemia cells with various ginger constituents galanals A and B (isolated from the flower buds of Japanese ginger) culminated in mitochondrial mediated apoptosis with a concomitant down-regulation of anti-apoptotic protein B-cell lymphoma-2 (Bcl-2) and an augmentation of pro-apoptotic protein Bcl-2-associated X protein (Bax). In another short term in vitro assay involving Epstein–Barr virus early antigen (EBV-EA) activation in Raji cells promoted by phorbol ester, TPA and ginger extract exhibited potent anti-EBV-EA activity. The rhizome extract, that exhibited EBV activation inhibitory activity, had no cytotoxic effect in Raji cells (Shukla and Singh, 2007 and refs. cited therein). The inhibitory activity of ginger extracts in tumor initiation and promotion is considered to be due to its pungent vanillyl ketones, including [6]-gingerol and [6]-paradol.
2.5.3.1. Skin cancer chemoprevention by ginger and [6]-gingerol

Anticancer potential of [6]-gingerol in skin cancer is quite well reported. Ultraviolet radiations from sun play an important causal role in skin cancer. Pre-treatment with [6]-gingerol abridged UVB-induced intracellular ROS levels, activation of caspase-3, -8, -9, COX-2 and Fas expression in HaCaT cells (Kim et al., 2007). Nuclear translocation of Nuclear Factor kappa B (NF-κB) from cytosol in HaCaT cells was suppressed by [6]-gingerol through suppression of inhibitor kappa B (IκB)-α phosphorylation (ser-32) (Kim et al., 2005; Kim et al., 2007) Cell transformation and activated protein (AP-1) activation induced by epidermal growth factor (EGF) in JB6 cells were also significantly inhibited by [6]-gingerol (Bode et al., 2001). [6]-Gingerol’s inhibitory effect on AP-1 transcriptional complex in human skin keratinocytes cell lines has also been reported (Davies et al., 2005).

In laboratory animals also ginger and its key pungent component [6]-gingerol have been demonstrated to inhibit promotion of skin carcinogenesis (Shukla and Singh, 2007 and refs. cited therein). [6]-Gingerol suppressed TPA-induced skin tumor promotion in addition to the inhibition of tumor necrosis factor (TNF)-α generation and epidermal ornithine decarboxylase (ODC) activity in ICR mice. Another study reported that the topical administration of the ethanol extract of ginger could significantly suppress TPA stimulated induction of ODC in SENCAR mouse skin. Significant inhibition of TPA caused epidermal edema (56%) and hyperplasia (44%) was provided by pre-application of ginger extract to mouse skin. Similarly, topical administration of [6]-gingerol also suppressed TPA-stimulated COX-2 expression with concomitant inhibition of NF-κB DNA binding activity in mouse skin (Shukla and Singh, 2007 and refs. cited therein). Topical application of [6]-gingerol (30 µM) preceding to UVB irradiation (5 kJ/m(2)) of hairless mice, has been reported to suppress NF-κB nuclear translocation, induction of COX-2, and p38 MAPK-NF-κB signaling pathway (Kim et al., 2005; 2007). [6]-Gingerol has been reported to have anti-tumorigenic potential in benzo[a]pyrene (BaP)-induced mouse skin tumorigenesis (Nigam et al., 2010). In a study by Surh et al. (1999) [6]-gingerol topical application prior to TPA was found to attenuate the skin papillomagenesis initiated by 7, 12 dimethyl benz(a)anthracene (DMBA) in female ICR mice. In human epidermoid squamous carcinoma A431 cells [6]-gingerol was found to induce apoptosis through ROS-mediated mitochondrial cell death pathway (Nigam et al., 2009a). Kim et al. have demonstrated that [6]-gingerol can prevent UVB-induced ROS production and COX-2 expression in vitro and in vivo (Kim et al., 2007).
2.5.4. Molecular targets of [6]-gingerol

Some of the molecular targets of [6]-gingerol against cancer have been illustrated in Figure 2.6. Studies have revealed that [6]-gingerol presents its anti-cancer properties through the inhibition of NF-κB. In an *in vitro* study, [6]-gingerol enhanced tumor necrosis factor related apoptosis inducing ligand (TRAIL)-induced apoptosis of cancer cells by suppressing TRAIL-stimulated NF-κB activation while [6]-shogaol alone induced apoptosis by damaging the microtubules (Ishiguro et al., 2007). Prior administration of [6]-gingerol inhibits NF-κB nuclear translocation from cytosol through suppression of phosphorylation (ser-32) of IκBα. EGF-induced AP-1 transactivation is inhibited by [6]-gingerol in a dose-dependent manner via blockage of EGF-induced AP-1 DNA binding activity (Bode et al., 2001).

![Figure 2.6](image)

**Figure 2.6.** Molecular targets of [6]-gingerol against cancers of different types. (Nigam et al., 2009)

Tumor angiogenesis is the formation of a network of blood vessels which infiltrate into cancerous growths, providing nutrients and oxygen, and, removing waste products.
Potent cell proliferation signals generated by various angiogenic growth factor receptors such as the EGF receptor, insulin-like growth factor (IGF-1) receptor, and vascular endothelial growth factors (VEGF)-receptor networks constitute the basis for receptor-driven tumorigenicity in the progression of several cancers (Aggarwal and Shishodia, 2006 and refs. cited therein). [6]-Gingerol inhibits angiogenesis by inhibiting both the VEGF- and Basic Fibroblast Growth Factor (bFGF)-induced proliferation of human endothelial cells and caused cell cycle arrest in the G1 phase. [6]-Gingerol also blocked capillary-like tube formation by endothelial cells in response to VEGF, and strongly inhibited sprouting of endothelial cells in the rat aorta and formation of new blood vessel in the mouse cornea in response to vascular endothelial growth factor (VEGF) (Kim et al., 2005; 2007). [6]-Gingerol stimulates apoptosis through upregulation of nonsteroidal anti-inflammatory drug-activated gene (NAG-1) and G-1 cell cycle arrest through downregulation of cyclin D1 (Lee et al., 2008).

Reports from our laboratory and others have earlier demonstrated that [6]-gingerol induces apoptosis in cancer cells via upregulation of p53, Bax, Caspase-9 and Caspase-3, and, downregulation of antiapoptotic proteins, Bcl-2 and Survivin (Kim et al., 2005; Park et al., 2006; Shukla et al., 2007).

COXs are prostaglandin-H-synthases synthesizing PGs from arachidonic acid released by membrane phospholipids. COX-2 is the inducible isoform of prostaglandin-H-synthase and is overexpressed in practically every premalignant and malignant condition (Subbaramaiah and Dannenberg, 2003). Depending upon the cell type and the stimulus, various transcription factors like AP-1, nuclear factor interleukin-6 (NFIL-6), NF-κB can induce COX-2 transcription (Aggarwal and Shishodia, 2006 and refs. cited therein). Lipopolysaccharide-induced COX-2 expression and prostaglandin E2 (PGE2) production are also inhibited by gingerols (Lantz et al., 2007). [6]-Gingerol has been reported to inhibit cell adhesion, invasion and motility in MDA-MB-231 human breast cancer cell lines by modulating the activities of MMP-2 and MMP-9 (Lee et al., 2008a).

TNF, originally identified due to its antitumor activity, has now been discovered to mediate processes of tumor initiation, promotion, and metastasis. Almost in all cell types TNF exposure activates NF-κB thereby inducing the transcription of inflammatory genes. As TNF has the critical role in mediating tumorigenesis, agents that can inhibit TNF activity have remarkable potential for therapy of TNF-linked cancers (Aggarwal and Shishodia, 2006 and refs. cited therein). [6]-Gingerol is one such agent capable of effectively suppressing TNF-α-induced c-Jun-NH(2)-terminal kinase (JNK) signaling activation (Isa et al., 2008).
2.6. Targeting cancer-associated genes by RNAi

Nowadays RNAi is being considered as a marvelous therapeutic strategy for cancer treatment because of its traits of high specificity and high sensitivity in binding gene products. For development of nuclear medicine against cancers, it is very crucial that appropriate gene targets are selected. Catalog of such targets includes antiapoptotic proteins, cell cycle regulators, transcription factors, signal transduction proteins, and factors related with malignant behavior of cancer cells. All these genes have been found to be linked with the poor prognosis of cancer patients.

Molecules of apoptosis/ cell cycle regulation are very important siRNA target candidates. The antiapoptotic Bcl-2 protein is found upregulated in many cancers and contributes in tumorigenesis and progression (Tsujimoto et al., 1985; McDonnell and Korsmeyer, 1991; McDonnell et al., 1992; Pezzella et al., 1993; Lu et al., 1993; Lauwers et al., 1995). Its overexpression correlates with the poor prognosis of cancer patients (Sinicrope et al., 1995; Pepper et al., 1996; Iqbal et al., 2006). Several studies have shown that treatment with siRNAs against Bcl-2 inhibited the cell proliferation in tumors. Cationic liposome-based delivery of synthetic Bcl-2 siRNA inhibited tumor progression in a xenograft mouse model (Yano et al., 2004; Fu et al., 2005; Sonoke et al., 2008). Survivin, a very important member of the inhibitors of apoptosis proteins (IAPs) family, performs a function in chromatin cleavage associated with spindle formation during cell division (Skoufias et al., 2000). Though its expression is almost undetectable in normal conditions (Ambrosini et al., 1997), overexpression has been shown in many cancers (Ambrosini et al., 1997; Altieri, 2003 b). This overexpression of survivin has shown good correlation with poor prognosis of cancer patients (Kawasaki et al., 1998; Monzo et al., 1999; Adida et al., 2000; Tanaka et al., 2000; Kamihira et al., 2001; Chakravarti et al., 2002). Furthermore, survivin is linked with resistance to chemotherapy and radiotherapy (Davidovich et al., 2004; Rodel et al., 2005; Tirro et al., 2006; Shi et al., 2007).

Another important class of siRNA target candidates comprises of signal transduction proteins. Signal transducer and activator of transcription (STAT) family members play key roles in cytokine signaling pathways that control gene expression (Darnell, 1997; Bromberg and Darnell, 2000). Out of the seven types of STATs, STAT3 is the one which is majorly implicated in carcinogenesis. In normal cells only transiently activated form of STAT3 is present whereas in many cancers a constitutively active form is detected (Bromberg, 2002) which dysregulates its downstream genes involved in cell proliferation and survival (Alas and Bonavida, 2001; Masuda et al., 2002; Konnikova et al., 2005). Inhibition of tumor growth and invasion has been observed where STAT3 protein levels
are depleted (Gao et al., 2005; Ling and Arlinghaus, 2005; Fan et al., 2008). Breakpoint cluster region-abelson (Bcr-Abl) fusion protein, produced by the molecular effect of the t(9;22) translocation, is a constitutively active tyrosine kinase that induces leukemias (Sawyers, 1999). Considering this causative role of tyrosine kinase in leukemias Imanitib mesylate (IM; Gleevec, Glivec) was developed as tyrosine kinase inhibitor and this development significantly improved the therapy outcomes of Philadelphia (Ph)–positive leukemia, especially chronic myelogenous leukemia (CML) (Sawyers et al., 2002; Talpaz et al., 2002; O'Brien et al., 2003; Druker et al., 2006). Since then many second generation TKIs have been developed to overcome resistance to IM and they have provided superb outcomes (Golas et al., 2005; Kantarjian et al., 2006; Kimura et al., 2006; Talpaz et al., 2006). All these findings suggest that Bcr-Abl protein is a promising target to abolish Bcr-Abl-positive leukemic cells and this contemplation is being investigated in vitro by knocking down the expression of Bcr-Abl mRNA by RNAi (Wilda et al., 2002; Li et al., 2003; Scherr et al., 2003). Additional studies are required for its clinical application.

β-catenin, a downstream protein of Wnt signaling pathway, has been reported to play key roles in various processes of development, proliferation, and differentiation (Wodarz and Nusse, 1998). When Wnt is absent multiple proteins, such as glycogen synthase kinase- 3, casein kinase 1 and adenomatous polyposis coli regulate the intracellular levels of β-catenin by phosphorylating it. Phosphorylated form of β-catenin is then degraded by the ubiquitin proteasome pathway. While in the presence of the Wnt ligand, β-catenin gets stabilized in the cytoplasm and is subsequently translocated to the nucleus where it binds to the T cell factor transcription factor and target genes such as cellular myelocytomatosis (c-Myc) and Cyclin D1, which are then upregulated thereby resulting in cell proliferation (Fodde and Brabletz, 2007). β-catenin overexpression has been observed in many cancers (Gamallo et al., 1999; Wong et al., 2001; de la Taille et al., 2003; Ysebaert et al., 2006; Ashihara et al., 2009). Knockdown of β-catenin by using specific siRNAs efficiently inhibited the proliferation of colon cancer cells and myeloma cells and induced caspase-dependent apoptosis (Verma et al., 2003; Ashihara et al., 2009).

Other siRNA target candidates are molecules that define the behavior of cancer, for instance metastasis or resistance towards cytotoxic agents. P-glycoprotein (P-gp) which is a product of multidrug resistance (MDR1) gene is overexpressed in many cancers and this overexpression of P-gp induces cross-resistance towards many structurally unrelated cytotoxic agents (Mahon et al., 2003). P-gp depletion in cancer cell lines by MDR1 siRNA was found to reverse the sensitivity to adriamycin or vincristine (Chen et
Additionally, chemosensitivity was enhanced in cancer cells by treatment with siRNAs targeting other molecules linked with chemoresistance, such as multidrug resistance protein 1 (MRP1), ATP-binding cassette superfamily G (White) member 2 (ABCG2), and MRP7/ ATP-binding cassette transporter 10 (ABCC10) (Leslie et al., 2001; Priebsch et al., 2006; Oguri et al., 2008).

As tumor cells need a rich blood supply and to achieve this, angiogenesis process is stimulated, another effective cancer therapeutic approach might be to knockdown genes that are involved in angiogenesis. The vascular endothelial growth factor (VEGF)/VEGFR axis performs an important function in angio- and lymphangiogenesis. VEGF-A induces angiogenesis in tumor vessels, increases permeability of blood vessels, and enhances cancer cells’ motility, thereby resulting in metastases (Yancopoulos et al., 2000; Ellis, 2004; Brader and Eccles, 2004). VEGF-A siRNAs treatment very effectively prevented cancer metastases (Liu et al., 2002; Guan et al., 2005a; Wang et al., 2008). VEGF-C is involved in tumor lymphangiogenesis and lymph node metastasis, and VEGF-C/D overexpression in cancer cells promoted metastases through lymph vessels (Skobe et al., 2001; Renyi-Vamos et al., 2005; Boone et al., 2008; Sugiura et al., 2009). Treatment with VEGF-C siRNA suppressed metastasis of breast cancer in a mouse xenograft model (Chen et al., 2005).

Besides inhibiting the expression of normal genes that are necessary for cancer cell growth and survival, RNAi can also be employed to target cancer-causing mutations. For example, dsRNA was transfected to target the M-BCR/ABL fusion site to annihilate leukemic cells possessing such a gene-rearrangement (Wilda et al., 2002). Leukemic cells lacking BCR/ABL rearrangement were not harmed by M-BCR/ABL-dsRNA.

One of the important siRNA targets is Plk1 which performs key functions in the regulation of mitotic progression, including mitotic entry, spindle formation, chromosome segregation, and cytokinesis. We have selected Plk1 for our study and review of its associated literature is given below.

### 2.6.1. Polo like kinase-1

Polo-Like Kinases (Plks) belong to the family of serine/threonine kinases and are highly conserved amongst eukaryotes. Plk family thus far comprises of four identified members viz. Plk-1, Plk-2, Plk-3, and Plk-4 in mammalians and Plks are regulators of both cell cycle progression as well as cellular responses to DNA damage (Clay et al., 1993; Barr et al., 2004; Eckerdt et al., 2005; Winkles and Alberts, 2005). Best characterized among the four Plks identified to date is Plk-1. Plk-1 has a serine/threonine protein kinase at N
terminal while the C-terminal region has two polo box domains that regulate the kinase activity of Plk-1 (Jang et al., 2002; Strebhardt and Ullrich, 2006). Plk-1 regulates cell division at many points in the mitotic phase: mitotic entry by activating cyclin dependent kinase 1 (CDK 1), formation of bipolar spindle, alignment of chromosomes, chromosome segregation, and cytokinesis (Barr et al., 2004; van de Weerdt and Medema, 2006). Plk-1 gene expression is very well regulated throughout the cell cycle progression, with the peak levels reaching at M phase (Lake and Jelinek, 1993; Lee et al., 1995). Plk1 protein also has a similar pattern of expression during various cell cycle phases and when cells exit from mitosis, Plk-1 gets degraded by the ubiquitin-proteasome pathway as it is a substrate of ubiquitin-ligase, known as the anaphase-promoting complex/cyclosome (Lake and Jelinek, 1993; Lee et al., 1995; Ferris et al., 1998; Alvarez et al., 2001; Lindon and Pines, 2004). In actively proliferating cells of tissues like placenta, spleen, and testis Plk-1 is highly expressed, while in most other adult tissues Plk-1 is scarcely present (Lake and Jelinek, 1993; Golsteyn et al., 1994; Hamanaka et al., 1994). Several reports have shown that Plk-1 is overexpressed in tumor tissues and that expression levels of Plk-1 were highly correlated with histological grades of tumors, clinical stages, and prognosis of cancer patients (Reagan-Shaw and Ahmad, 2005; Kawata et al., 2008). In non-small cell lung cancer (NSLC) tissues Plk-1 mRNA levels were up-regulated and correlated with the survival rate of patients (Wolf et al., 1997). Immunohistological study also demonstrated that Plk-1 protein was elevated in NSLC tissues in patients having progressed stages of cancer (postsurgical stage II) and in patients with poorly differentiated NSLCs (Kawata et al., 2008). Urinary bladder cancer patients having high levels of Plk-1 were found to have poor prognosis in comparison to patients with its low expression. Histologically high-grade, deeply invasive, lymphatic-invasive, and venous-invasive bladder cancers were observed to have significantly elevated Plk-1 expression (Nogawa et al., 2005). Plk-1 overexpression is observed in various cancers and its overexpression is a prognostic biomarker for cancer patients (Table 2.4.).

**Table 2.4. Plk-1 Overexpression is Correlated with the Prognosis of Cancers Patients.**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Cancer Type</th>
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<tbody>
<tr>
<td>1</td>
<td>Oropharyngeal carcinoma</td>
</tr>
<tr>
<td>2</td>
<td>Esophageal carcinoma</td>
</tr>
<tr>
<td>3</td>
<td>Non-small cell lung cancer</td>
</tr>
</tbody>
</table>
Suppression of Plk-1 activity stimulates mitotic arrest and apoptosis in tumor cells (Spankuch-Schmitt et al., 2002; Gumireddy et al., 2005; Steegmaier et al., 2007). Cells transfected with Plk-1 siRNA had dumbbell-like and misaligned nuclei, suggesting that Plk-1 depletion inflicted anomalies of cell division, and these cells then progressed to caspase-dependent apoptosis (Liu and Erikson, 2003; Nogawa et al., 2005; Kawata et al., 2008). Plk-1 knockdown with siRNA treatment arrests the cells at the G2/M phase of cell cycle with a concomitant increase in Cell Division Cycle 2 (Cdc2)/ Cyclin B1 proteins (Liu and Erikson, 2003; Reagan-Shaw and Ahmad, 2005; Nogawa et al., 2005; Kawata et al., 2008; Judge et al., 2009). Inhibition of cell proliferation by Plk-1 silencing has been demonstrated in various malignancies including prostate cancer (Reagan-Shaw and Ahmad, 2005), esophageal cancer (Bu et al., 2008), hepatocellular carcinoma (Judge et al., 2009), breast cancer (Spankuch et al., 2007), gastric cancer (Chen et al., 2006b) and leukemia (Renner et al., 2009; Ikezoe et al., 2009). In addition, preclinical studies in xenograft mouse models also showed that the administration of siRNA against Plk-1 inhibited the growth of cancers (Spankuch et al., 2004; Nogawa et al., 2005; Guan et al., 2005b; Spankuch et al., 2006; Kawata et al., 2008; Judge et al., 2009).

Depletion of Plk-1 enhances the sensitivity of cancer cells to many anticancer agents (Spankuch et al., 2007; Yu et al., 2008). Polymerized microtubules append to kinetochores and their subsequent dynamics leads to cell division (Jordan and Wilson,
2004). Paclitaxel (PTX) acts as an anticancer agent by targeting microtubule dynamics (Jordan and Wilson, 2004; McGrogan et al., 2008). Plk-1 is important for this microtubule assembly and its knockdown by siRNA downregulated the expression of spindle checkpoint proteins mitosis arrest deficient 2 (Mad2), centromere protein E (Cenp-E), highly expressed in cancer (Hec)/Ndc80, and spindle checkpoint (Spc) (Ahonen et al., 2005). Combination treatment of Plk-1 siRNA with paclitaxel synergistically suppressed the proliferation of breast cancer cells (Spankuch et al., 2006; 2007). p27 kinase-inhibitory protein 1 (kip 1), cyclin-dependent kinase inhibitor (CDKI) regulating G0 to S phase transition, is an atypical tumor suppressor and its reduced expression has shown correlation with poor prognosis of cancer patients (Sherr and Roberts, 1999; Chu et al., 2008). Herceptin, a humanized monoclonal antibody against human epidermal growth factor receptor 2-positive cancers, is an important drug for treatment of breast cancer. p27 kip1 expression is down-regulated in herceptin-resistant breast cancer cells and administration of exogenous p27 kip1 enhanced herceptin sensitivity (Nahta et al., 2004). Plk-1 siRNA acts synergistically with herceptin via inducing p27 kip1 expression and facilitating the induction of apoptosis by the cleavage of caspases in HER2-positive breast cancer cells (Spankuch et al., 2007).

Although Plk-1 performs vital roles during the course of cell division, its depletion does not upset the proliferation of normal cells (Spankuch-Schmitt et al., 2002; Liu et al., 2006b; Kawata et al., 2008). This indicates the presence of some other kinases which can compensate the absence of Plk-1 function in normal cells (Liu et al., 2006b; Kawata et al., 2008). As depletion of Plk-1 provokes mitotic catastrophe and cell death in cancer cells specifically without affecting the normal cells, Plk-1 promises to be an excellent target for cancer therapy.

2.6.2. RNAi in chemosensitization

Radiation therapy and chemotherapy are two important modes of cancer treatment. Although a number of chemotherapeutic treatments have been shown to be effective at inhibiting or eliminating cancer cell growth in preclinical studies, clinical applications are often limited due to the toxic side effects associated with anticancer drugs. Many of those therapeutic agents used in the clinic have the ability to induce the DNA damage; however, they may also be highly cytotoxic, causing peripheral toxicity and secondary cancer as adverse side effects. Patients are often unable to tolerate the level of a drug needed to effectively eliminate malignant cells while levels that can be tolerated are insufficient therapeutically. As a result, chemoresistance and subsequent tumor recurrence are often the outcome of such therapies. Novel targeted therapies that
interfere with specific molecular signaling pathways affecting cancer cell survival are being developed as potential treatment options to render cancer cells more sensitive to cytotoxic chemotherapy. Such therapies have the potential to increase drug efficacy while reducing toxic effects on untargeted cells. Targeted cancer therapy by RNAi is a relatively new approach that is being employed for chemosensitization of cancer cells towards chemotherapeutic agents.

X-linked IAP (XIAP) is an important member of inhibitor of apoptosis (IAP) family. Wang et al. (2008) explored the effect of knocking down XIAP on the proliferation, apoptosis and radiosensitivity of human laryngeal carcinoma cells (Hep-2). A siRNA expression vector (pSilencer4.1-XIAPshRNA) specifically targeting XIAP was transfected into Hep-2 cells, and then the clonogenic cell survival assay was conducted to determine the effect on radiosensitivity of Hep-2 cells. Results showed that the up-regulated levels of XIAP were linked with resistance of human malignancies towards radiotherapy and depletion of XIAP expression could significantly enhance radiosensitivity in laryngeal carcinoma cells. Thus, this study indicated that combinatorial therapy with XIAP inhibition and radiation can be an effective strategy for cancer treatment (Wang et al., 2009).

Paclitaxel (PTX) is one of the most efficacious chemotherapeutic drugs for cancer that represses dynamic instability of spindle microtubules and inhibits mitosis by binding to β-tubulin, thereby inducing G2/M arrest and apoptosis. It is currently being used at clinical level for the therapy of many solid tumors including ovarian, breast, prostate, nonsmall-cell lung cancers (Rowinsky, 1997), and also in treatment of advanced human cancers which are resistant to conventional chemotherapy. But, in spite of this good efficacy as a chemotherapeutic agent usage of PTX is often limited because many tumors develop resistance towards PTX. Earlier studies have shown that proteins like Phosphatase and Tensin Homolog Deleted On Chromosome-10 (PTEN), Phosphoinositide-3 kinase (PI3K), Akt and MDR protein form a complicated signaling network which underlies behind ovarian cancer PTX resistance. Recent evidence has indicated that Akt2 plays a crucial role in protecting cells from PTX-induced apoptosis (Yuan et al., 2003; Cheng et al., 2007; Xing et al., 2008). Weng et al. (2009) employed RNAi-mediated silencing approach to explore the role of Akt2 pathway on PTX-resistance in ovarian cancer cells and showed that Akt2 knockdown sensitized ovarian cancer cells to PTX-induced apoptosis, and inhibited survivin expression.

Though 5-Fluorouracil (5-FU) is an effective chemotherapeutic agent for nasopharyngeal carcinoma (NPC) (Lin et al., 2003; Chan et al., 2005; Wee et al., 2005) it induces drug
resistance after several cycles of 5-FU-based chemotherapy (Mader et al., 1998). The oncogene B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI-1) has been reported to protect cancer cells from apoptosis (Hannon, 2002; Liu et al., 2006a; Kang et al., 2007). When 5-FU treatment was given in BMI-1/RNAi-transfected cells the percentage of apoptotic NPC cells were found to be higher than that among cells transfected with the empty vector. Significant reduction in 50% inhibitory concentration (IC50) values of 5-FU was recorded in the cells transfected with BMI-1/RNAi (Qin et al., 2008). Additionally, the expression levels of phospho-AKT and the anti-apoptotic protein Bcl-2 were reduced in the cells having depleted levels of BMI-1, whereas the levels of apoptosis-inducer Bax were upregulated. Inhibition of AKT pathway by a PI3K inhibitor could not further enhance the 5-FU sensitivity in the cells having reduced levels of BMI-1 expression. Thus, the results suggested that BMI-1 depletion enhanced the 5-FU chemosensitivity in NPC cells by inhibiting PI3K/AKT pathway and combining 5-FU treatment and BMI-1 depletion can be an effective strategy for cancer chemotherapy (Guo et al., 2007).

2.6.3. RNAi clinical trials towards cancer therapies

For a newly discovered technology, siRNAs have entered the clinics at an incredible pace. The first systemic administration of a siRNA drug for cancer treatment in humans was conducted in a CML patient (Koldehoff et al., 2007). A 47-year-old, Ph-positive and IM resistant, female CML patient received allogeneic bone marrow transplantation but an extramedullary relapse of pleural effusions and subcutaneous nodes cropped up. Then the patient was given further intravenous or subcutaneous treatment of siRNA targeting Bcr-Abl mRNA. This study showed that the siRNA treatment was quite well endured without any adverse consequences.

Though several siRNA therapies for various diseases have been performed in clinical settings, but only a few clinical trials for cancer treatment are ongoing (http://clinicaltrials.gov/ct2/home) as most of the present siRNA therapeutics in advanced clinical trials rely on localized drug delivery (Behlke, 2008). Alnylam Pharmaceuticals is developing RNAi therapeutic ALN-VSP01 against kinesin spindle protein and VEGF, and carrying out its Phase I trial in patients with advanced liver cancer. Calando Pharmaceuticals has started a Phase I trial of CALAA-01 in patients having solid tumors refractory to standard-of-care therapies. CALAA-01 targets a subunit of ribonucleotide reductase, an enzyme involved in DNA synthesis. This is the first study where receptor-mediated delivery of siRNAs is being exploited. In CALAA-01 siRNAs are encapsulated in transferrin-attached cyclodextrin particles which promotes siRNA uptake by cells that
express transferrin receptor, which is highly expressed on cancer cell surfaces. CALAA-01 has been proved safe and efficient in mice and nonhuman primates (Heidel et al., 2007; Bartlett and Davis, 2008).

Clinical trials employing Locked Nucleic Acid (LNAs) are also being carried out. Santaris Pharma has developed LNA called SPC2996 against Bcl-2 and its Phase I/II trial in patients with relapsed or refractory chronic lymphocytic leukemia is being conducted. Enzon Pharmaceuticals has developed a LNA targeting hypoxia-inducible factor-1α and are conducting Phase I/II study in patients with advanced solid tumors or lymphoma.