

INTERACTION AND CYTOTOXICITY OF COMPOUNDS WITH HUMAN CELL LINES

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(Received January 10, 2014)

The interactions of compounds on human cell lines are either influenced by the composition of substances present in plant material or alteration of constituents by solvent fractionation. These substances or constituents have an influence on the percentage cytotoxicity readings of compounds in human cell culture. Understanding and correlating the relationship between cytotoxicity and other parameters, such as cell death inducing mechanisms, will assist pharmaceutical chemists to synthesize compounds that can target particular ailments with greater efficiency. This will also allow scientists to understand the interaction of compounds with different cell types for different compound fractions.

Keywords: cytotoxicity, interaction, tumour and normal cell lines.

INTRODUCTION

The interaction and cytotoxicity of compounds with different cell lines in human cell culture is often reported exhibiting relationships of concentration dependence or independence as important determinants for drug design in the pharmaceutical industry and industries aimed at targeting infected or tumour cell lines like cancer, Human Immunodeficiency Virus and tuberculosis. With the developments of modern biotechnology, these relationships that aim at protecting the cells of the human body can be confirmed through attempts of using alternative methodologies with or without any success. The methodologies used to test the cytotoxicity of compounds and to understand their interaction with various types of normal and infected cells have been used for almost four decades in search of therapeutic cures in the form of modalities, and revert back from traditional and herbal medicines used by rural communities in different parts of the world, such as China, Japan, Asia, South Africa and India (1). All these countries are in direct need of improved verification methods and relied on methods that either involve drug-susceptibility testing or screening particular genes within cell lines exposed to

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compounds as verifiers of drug susceptibility or resistance. These techniques, as we know it, are well established in scientific practices (2). The development of DNA technology since the mid 1800s aimed precisely at sequencing DNA and understanding the molecular genetics of prokaryotic to eukaryotic genomes, as researched by James Watson and Francis Crick in 1953 (3). During the latter part of this century, Mossman developed a technique: MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) cytotoxicity assay, which he used to screen the toxicity of compounds (4). Since then, many other techniques have been developed to screen compounds from prepared formulations or materials extracted from plant parts. The mentioned methods, as well as those that follow, measure the cytotoxicity of compounds in human viable cell lines. Methods include the colony formation method, CCK-8 (cell counting kit-8), crystal violet method, XTT (2,2-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay, ⁵¹Cr (chromium) method, trypan blue assay, tritium-labelled thymidine uptake method and the WST (water-soluble tetrazolium salt) methods (5). The development of technologies to quantitate the cytotoxicity of compounds allows scientists to understand the behaviour of cell lines under stress conditions that are induced by the constituent components of compounds within the pharmaceutical, medicinal and therapeutic industries.

The MTT cytotoxicity assay has been used more frequently compared to the XTT method because of its ability to reproduce results rapidly. However, it is less reliable. These two assays improved the indulgence for screening the cytotoxicity of compounds in general, and it enabled modalities to be manufactured at potencies that are beneficial for human consumption (6). However, the problem with screening compounds is that their interaction in human cell culture is variable and they have not been reported at a predictable level, which has further emphasized the search for newer methods. The variable interactions of human cell lines to compounds have made it virtually impossible to obtain standard cytotoxicity result sets for specific cell lines and for specific types of compounds. This has created a hindrance for commercialising and manufacturing them.

The majority of studies that report cytotoxicity results do not appreciate that it is actually a measure of mitochondrial activity in viable cell lines. This fact is normally unnoticed during the interpretation process, and some are often reported as purely cytotoxic or proliferative. Therefore, cytotoxicity also has an effect on the interaction of compound on the cell line, and the behaviour of the cell type may be influenced by the cytotoxic level of the compound (7). Various cell signalling events that involve the expression of cell death or growth proteins, like bid, bax, and bcl-2, become activated when cell lines are exposed to compounds (or stimuli) (8). Their exact role (either growth or death) is determined by the level of compound uptake by viable cells, and the cytotoxicity of the compound thereof (9). Overall, the dose-and-time response of cell line to compounds is thus unestablished, and it is uncertain whether they are conclusive at all. However, what

is known is that many papers report dose-and-time responses as well as concentration dependent relationships that are justifiable. These relationships, however, have become confusing over a few recent studies that have shown cell lines behaving unfavourably to particularly studied compounds. The question that arises from such situations is whether those cytotoxicity results are valid, and if so, what techniques are available to confirm them reproducibly and accurately? This review will discuss important outcomes in cytotoxicity results and will consider the relationships between the interaction of human cell lines and compounds/stimuli that have possibly produced those results.

HUMAN CELL CULTURE – NOW AS BEFORE

Over the last 62 years, human cell culture has been a great method for studying normal and infected cells *in vitro*. This development of studying cell lines *in vitro* arose ever since George and Margaret Gey, from John Hopkins University, discovered a way of avoiding arrested cell proliferation outside the human body. Over the years, the development of human cell culture has thus increased the number of studies conducted without gambling with patient lives. The problem was that they were unable to keep a lineage of cultured and sub-cultured cells alive and so, with the help of a research assistant, Mary Kubicek, the Geys managed to find a way of achieving this (10). The first human cell culture to have been successfully kept alive was named the HeLa cell line. It was named then after, the now late, 31 year old Henrietta Lacks, the patient whose cell line still continues to help scientists all over the world even today. The cell line was found to have a high level of proliferation, reaching a confluency of 100% in just 4 days. This is the earliest and most valuable discovery in human cell culture, even at present (11). The introduction of other cell lines such the fibroblasts (normal cells), HEP-2 (laryngeal cancer) and K562 (liver cancer), to name just a few, are measured for their robustness and *in vitro* multiplication rate relative to the HeLa cell line at the time of discovery and today, when reporting cytotoxicity percentages for example (12). Those cell lines are therefore inferred relative to the HeLa cell line in respect to their morphological characteristics and genetic variability and are used as derived cell lines from that discovery.

The earliest and most recently discovered human cell cultures follow specific protocols during experimentation, and these depend on whether the cell lines used are adherent or in suspension, especially since adherent cell lines (for example HEP-2) require an additional trypsin-detachment before experiments like growth proliferation, arrest and inhibition assays are performed (13). For decades, various attempts at studying cells accurately *in vitro* have been developed and successful results on the interaction of cell lines with plant-derived or natural compounds have been reported quite extensively in the literature. It has been known for many years

that all eukaryotic cells undergo the same phases of division, irrespective of their genetic machinery, and these phases include the mitotic phase divisions, such as interphase, prophase, metaphase, anaphase and telophase. These phases will not be discussed in detail, but what is important to know are the subdivisions of interphase: G1 (growth phase), S (replication) and G2 (growth phase) phases. With the interplay and marriage of different phases in human cell culture, and the interaction of these phases with compound treatment, it is possible to predict that in time, the development of drugs to target specific phases of the cell cycle may be far more beneficial to the treatment of ailments and/or diseases in patients than any of the current modalities and scientific methodologies that prevail (14).

THE HERBAL INDUSTRY

Herbs represent the stagnant backbone of health care in many developing countries, both in terms of their traditional value and application in homeopathic medicine as well as for domestic remedies used by rurals on the external of the body to treat burns and wounds, for example (15). In China, India, Japan, Pakistan, Sri Lanka and Thailand, traditional medicine is practiced widely, because of the rich store of herbal medicine found in the legumes of plants that belong to the families *Caesalpiniaceae*, *Fabaceae* and *Mimosaceae*. In Africa, the plant mostly used traditionally is *Phytolacca dodecandra*, mainly for the control of schistosomiasis, amongst others, which are less commonly used and are restricted to parts of Botswana, Cameroon, Egypt, Lesotho, Namibia, Madagascar, Mozambique, Nigeria, Rwanda, South Africa, Sudan, and Zaire. In the USA, the plant species *Eupatorium perfoliatum*, *Podophyllum peltatum* and *Panax quinquefolium* are used by American Indians because of their ornamental value (16). All plant material contains phenol compounds, which are secondary metabolites that belong to a large heterogeneous group of biologically active non-nutrients, in addition to flavonoids. These compounds provide various properties to the herbal industry, such as anti-inflammatory, immune modulating, antitumour, antiviral, antibacterial, antifungal and anti-oxidant (reactive oxygen species) (17, 18). Constantly, new plants are being discovered and screened for their chemotherapeutic value on infected cells relative to control cell groups such as macrophages, fibroblasts and red blood cells. This also implies to some older 'unnoticed' plants that are believed to be therapeutically beneficial.

Secondary metabolites are synthesized by plants when their morphological and biochemical patterns are interrupted by pathogens, wound effects and adverse environmental conditions, and this encumbers the production of phenolic substances in plant material (19). Therefore, *in vitro* toxicity of plant material on human cell culture, and the existing reports of such on polar and non-polar fractions of herbal material are of some concern regarding the reproducibility of data sets in different parts of the world, resulting in questions being prompted on

the methodologies utilized. In particular, the interaction of human cell culture with different plant fractions (of different organ cultures) is appraised, but their ability to bring about the same effect several times at different periods has also been one of the recent developmental questions when performing human cell culture and cytotoxicity experiments. To save scientific resources, the fractionation process of plant extracts used in growth inhibition, proliferation and arrest assays has become shortened by following dilution series and dose-and-time response patterns in contemporary years. Such relationships create problems in pharmaceutical industries when attempts are made to reproduce them fail. Therefore, understanding the factors that contribute to particular interactions and cytotoxicity results on human cell culture are important to elevate this problem.

Recently there have been several examples about the interaction of cytotoxic compounds on human cell culture, which include information on the constituent compounds that enhance or inhibit their activity on known cell cultures under standard laboratory conditions, while their preparation procedure is the same as the compound themselves (20). One well known example is the cytotoxicity of the isolated compounds (cinnamic acid: 5, 10 and 25 $\mu\text{g}/10\ \mu\text{l}$ of cells; caffeic acid: 5 $\mu\text{g}/10\ \mu\text{l}$ of cells) from the plant *Baccharis druncunculifolia*. *B. druncunculifolia* is an important vegetal source of propolis in southeast Brazil, found to inhibit HEP-2 cell growth at lower concentrations, while at higher concentrations it was found to enhance HEP-2 cell proliferation (21). Herbal material displaying cytotoxic activity at higher concentrations is taken to be obvious, and has been often reported in the literature, inclusive to being confirmed by many related studies. Furthermore, in the *B. druncunculifolia* study, Búfalo and co-workers found that the propolis had a much greater cytotoxicity on the HEP-2 cell line compared to extracts prepared from the actual vegetal source. This revelation confirms the belief that secondary metabolites have a huge influence on the outcome and interaction of compounds in human cell culture, and that fractionation could possibly have an impact on the compounds in the plant material.

The combination of plant organs and analysis of different extracts concurrently is common in human cell culture studies. When HEP-2 was treated with extracts and essential oils of *B. druncunculifolia* (5, 10 and 25 $\mu\text{g}/10\ \mu\text{l}$), the propolis, caffeic and cinnamic acid exhibited no cytotoxic effect. However, at higher concentrations (50 and 100 $\mu\text{g}/\text{ml}$ per 100 μl of HEP-2 cells) the propolis and vegetal source extracts had a concentration-dependent cytotoxicity. In contrast, studies showed that squiterpene and terpene phenols from *B. druncunculifolia* exhibit potent cytotoxic effects on leukaemia cells. These results indicate that there is a possibility of contradictory information about plants inducing varied cytotoxicity levels or proliferative effects in infected cells, and that this could be attributed to the different types of phenolic substances within the plant and prepared fractions. It also suggests that irregular cytotoxicity levels could be encountered in different human cell culture systems.

CELL DEATH-INDUCING MECHANISMS

The mechanisms by which eukaryotic cells die or lose viability are well recognized processes and provide many simplistic views about the biological nature in which the cell executes death under unfavourable conditions, such as in the absence of mitogens or growth factors. The mechanisms utilized vary and in isolation they are: apoptosis, autophagy, oncosis and necrosis.

Apoptotic cell death, also called programmed cell death, occurs when the genetic cell death machinery of a cell is induced. The term 'programmed' refers to a fixed pathway which dying cells follow; however, apoptosis can also be induced when cells are exposed to cytotoxic compounds (22). This process is characterised by morphological changes like cellular shrinking, condensation, chromatin margination, as well as the ruffling of the plasma membrane into apoptotic bodies (23). Similarly, like apoptosis, autophagy (macroautophagy, microautophagy, and chaperone-mediated autophagy) is genetically programmed and it has been observed in all eukaryotic cells ranging from yeast to mammals. Being an intracellular degradation system, it seizes the cytoplasm and organelles into double-membrane vesicles and traffics the contents to lysosomes for degradation. Recent studies have found that there is considerable overlap between apoptosis and autophagy, mostly because autophagy can enhance the possibility of apoptosis when over-induced (25). Furthermore, it has been found that autophagy can be induced in human cancer cells through chemotherapeutic agents (26). On the other hand, necrosis refers to natural cell death and the features that appear after a cell has died (27), while oncosis refers to any kind of cell death that is characterized by a marked cell swelling (28). Given that the subject matter under consideration is the cytotoxicity of compounds and their interaction in human cell culture, apoptosis would be discussed in more detail, since necrosis and oncosis are related, but are not induced by cytotoxic compounds.

When apoptosis is induced, a cascade of cellular events involving genetic activation occurs. *caspase-3* is the main molecular indicator of apoptosis, while several other genes that participate in the eukaryotic cell cycle, and possibly linked to autophagy, play a pivotal role before *caspase-3* activation. Some of these genes – *bid*, *bax*, *bcl-2*, and *apaf-1*. *bax* and *bcl-2* – are analogous genes and they control the level of apoptosis (or autophagy) within the cell. *bax* is the death-promoting gene, while *bcl-2* is the growth-promoting gene, a fact known for decades, and the event induced within cells depends on the degree of activation of either gene (29, 30). However, although *caspase-3* is the main indicator of apoptosis, its induction can involve any three pathways of apoptosis: receptor-ligand mechanism, mitochondrial apoptosis or endoplasmic reticulum apoptosis (31). These mechanisms will not be discussed in detail. However, what is important to know is that there is currently an increasing wealth of knowledge, and research being conducted, on cellular ligands to determine whether human cell cultures treated

with compounds could possibly die due to receptor-ligand and mitochondrial mechanism induction, since the two mechanisms are genetically interlinked. They are interlinked in the sense that induction of the receptor-ligand mechanism can trigger mitochondrial apoptosis. However, mitochondrial apoptosis cannot be triggered by receptor-ligand mechanism induction since it is possible for the stimulation of the mitochondrial apoptotic pathway to occur independent of the receptor-ligand mechanism. This means that it is not possible for the reverse process (mitochondrial apoptotic mechanism induction to receptor-ligand mechanism) to occur, since mitochondrial apoptosis does not have the ability to translocate compounds to the cellular receptor-ligands to induce the receptor-ligand apoptotic mechanism. Cellular ligand research offers numerous benefits to drug designers and developers in the infectious disease and cancer research fields, since it would allow drugs (or plant-derived compounds) that are specific to certain genetic markers, for specific apoptotic pathways, to be produced.

DOSE/CONCENTRATION AND TIME RESPONSES

When reporting cytotoxic and/or proliferative activity of compounds, researchers often search scientific literature for analyzing dose or concentration and time responses, and the usage (dilution or concentration series tested) for specific compounds tested in particular cell lines. A good example of dose/concentration dependence has previously been discussed in this review for *Baccharis drucunculifolia*. Other plant extracts that have been tested for their cytotoxicity include *Phytolacca dodecandra* and *Eupatorium perfoliatum* (16). However, there is a lack of interest in understanding the interaction of compound concentration with the cell type being studied and although all eukaryotic cells have the same cellular structure, recent studies have reported results which show that this opinion in determining the correct dose and time response is important for making conclusions about cytotoxicity and hence, rational drug design (32). Therefore, intense effort needs to be made to determine proper administration concentrations to cell lines for specific compounds.

Although mostly dependent relationships have been reported, studies have also reported time and dose independent relationships for cytotoxicity. For example, researchers found that the effect of purified ceramide on HEP-2, AMGM5 (Ahmed-Majeed-glioblastoma-multiforme-2005) and normal REFAM3 (rat embryo fibroblast) cells to have varying levels of cytotoxicity. They found a 1% cytotoxicity in HEP-2 cells for 7 μM of ceramide. When the concentrations were increased to 15 μM , 30 μM and 60 μM , the cytotoxic effect was 13.2%, 13.1% and 12.5%, respectively (33). This is evidence that at increasing concentrations, cell-death inducing mechanisms (usually apoptosis) have the ability to lose their full activation strength, causing death-promoting factors to become activated – hence, the cell growth effect. Researchers at the Durban University of

Technology also found that some cell lines are resistant to certain compounds and that some cells begin to multiply as a survival strategy.

A more prominent example of a dose independent relationship with time is the cell viability of RD (rhabdomyosarcoma) cells. It has been reported that at 7 μM and 15 μM ceramide, the HEP-2 cell percentage inhibition was 6.7% and 7.5% respectively. However, at 30 μM ceramide, a higher toxic effect (26.3%) was produced, with a steep reduction (18.8%) at 60 μM . Similarly in AMGM5-ceramide-treated cells, the % inhibition was 21%, 47% and 79% accordingly, when cells were treated with 7 μM , 15 μM and 30 μM of the compound. At 60 μM ceramide, the inhibition percent decreased to about 50%. This implied that cells treated with 30 μM ceramide could have probably suffered a significant reduction in cell viability (33).

As already mentioned, plant-derived compounds contain a cocktail of components and/or substances. Secondary metabolites, flavonoids and phenolics are major plant substances that have an influence on the desired cytotoxic effect when extracts or fractions are made from plant materials. This could have been the case with extracts from *Platycodon grandiflorum* that exhibited both cytotoxic and proliferative ability (34). In relation to the examples involving ceramide as an apoptotic and cytotoxic stimulus, it is known that certain plant fractions and normal compounds exhibit their cytotoxic effect over a certain threshold only, based on their ability to generate compounds that are specific to cellular ligands to induce cell death (35). For example, ceramide induces cytotoxicity in HEP-2 because of their ability to generate ceramide-1-phosphate by the action of an enzyme, ceramide kinase (36). This occurs because ceramide is an important cell membrane component that is hydrolyzed by phosphatase (37). Researchers infer that ceramide-1-phosphate can form intracellularly (38). Therefore, it could be postulated that ceramide-containing plants may play a greater role in cell proliferation because they may bind to CD95 receptors and inhibit apoptosis by initiating cell proliferation and senescence (39). Huge scientific efforts are thus needed to justify and clarify these relationships for different cell types and compounds, with an emphasis and need to work towards improving the current modes of therapeutic action and methodologies used to test the cytotoxicity of administered compounds.

SELECTIVE CYTOTOXICITY/PREFERENTIAL UTILISATION OF SPECIFIC COMPOUNDS BY CELL LINES

In cytotoxicity studies, although selective cytotoxicity of compounds or responses of human cell culture to cytotoxic stimuli are reported, this concept has not been properly explained. This concept, although widely understood, puts the clarity of currently available cytotoxicity reports under considerable strain, because the interactions of specific compounds (within extracts or natural compounds) on

particular human cell culture types have no specificity really, mainly because either plant material is exposed to environmental pressures or the material is collected from different localities (climatic variation) (40). Apart from this fact, one of the most important findings from studies is that the treatment of human cell culture with plant fractions (extracts) may not necessarily be dose and time dependent (as mentioned previously), due to the presence of phytochemicals that are attributed to secondary metabolites mostly and which have potential chemopreventative or chemotherapeutic efficacy against various types of infected cells (41). Secondly, although not clear, selective cytotoxicity has been indirectly and widely-researched in human epithelial cell lines like colon, laryngeal and cervical cancer (42). However, one problem is that cell lines derived from cervical cancer cells (HeLa) may resist phenotypic and genotypic changes to growth medium chemicals, which may contribute to cytotoxicity percentage irregularities that are reported as being selective (43). The HEP-2 cancer cell line typically exhibits this response, due to its hardiness derived from HeLa cell contamination (44). A study investigating the cytotoxicity of 3 *Fusarium* mycotoxins on HEP-2 and HeLa cells (using the MTT cytotoxicity assay) justifies such a response. Calvert and co-workers found HeLa cells to be most sensitive after two days of T-2 toxin and zearalenone utilisation at 0.1 µg/ml and 1.0 µg/ml administered concentrations, respectively. Furthermore, after further two days, rapid cell death was induced by 0.2 µg/ml deoxynivalenol toxin. This is a case in which the HeLa cell line has a selective cytotoxicity for each of the three compounds, while noting that rapid cell death was not induced by zearalenone at 1.0 µg/ml (higher concentration) (45). This has a profound effect on the future of reporting cytotoxicity results for chemotherapeutic value in industry, and probably needs to be given greater recognition by working and research scientists in laboratories. Following that justifiable experiment, the study further indicated the sensitivity of the HEP-2 cell line to deoxynivalenol after 2 days of exposure at 0.1 µg/ml, with complete induction of cell death at the same time-frame with a concentration ten times the sensitivity concentration (1.0 µg/ml). However, the intriguing point here is that at 1.0 µg/ml, cell death induction was noted after two days exposure. Hence, at day four, a lower concentration of deoxynivalenol induced HEP-2 cell death, while 2 days prior to this cytotoxicity reading, a concentration 10 times higher was required to induce cell death. This indicates that the cytotoxic effect of mycotoxins and other fraction possibility types may be time dependent for utilization and concentration independent. Studies have attributed this to the absence of autocrine apoptosis in HEP-2 cells during the depletion of growth medium chemicals (upon cellular utilisation and mitochondrial respiration). Furthermore, the response of cell types to stimuli depends on the replication machinery of the cell line upon induced proliferation/cytotoxicity. In colon cancer, Calvert and co-workers found all 3 mycotoxins to have no cytotoxic effect, which indicated some type of cell-type mycotoxin specificity. The same for other compounds have not yet been reported. The reporting of selective

cytotoxicity is important in standardizing laboratory protocols and understanding the response of cell lines to different chemical stimuli. Furthermore, authors have added and increased the complexity of this concept by suggesting that compounds may be less cytotoxic to mammalian cells and on occasion, the same compounds could enhance the cleavage activity of cell types, the latter referring to cell proliferation (45).

REACTIVE OXYGEN SPECIES

The contribution of reactive oxygen species to cell death (apoptosis or necrosis) in human cell culture is just as established as not having an effect on human cell culture. The MTT cytotoxicity assay has been used mostly to conduct cell proliferation/ death assays of various compounds for different human cell/tissue culture systems, because of its rapidity to reproduce results (46). However, in all of the assays it is important to realize that cell lines are usually viable and respiring, and confirmation in the MTT is achieved through formazan crystal formation. Only after a period of incubation for different cell types does the exposure of human cell culture to compounds results in increased respiration (more cells grow) or the adverse effect (cytotoxic effect) in infected cells, for example (47). But where does reactive oxygen species fit into this situation? And how does the respiration process contribute to reactive oxygen species effects? The answer is simple and a case for justifying cytotoxicity results in either dose and time, selective cytotoxicity or concentration independent studies.

Reactive oxygen species are important and of particular interest during the discovery of new compounds from plants, or when plants are being assayed for cytotoxic compounds in infected cells, because the respiration process upon utilization can contribute additionally to the percentages achieved for particular fractions (48). Hypoxia and hypotension during cellular respiration have been reported minimally during the past century, mainly because of the rapidity in achieving scientific data and publishing them in literature – as platform for further investigations mostly, which is understandable. However, it is obvious that investigating this fact could cloud the presently available cytotoxicity data sets, by increasing their unreliability. Within plant extracts, the presence of reactive oxygen species may enhance the hypoxic effect or contribute toward the acceleration of cell death or injury (48). The latter events may occur due to lipid peroxidation of cell membranes, protein and DNA oxidation, that specifically targets the pathways of apoptosis, and which probably lends itself to any of the three autophagic modes of cell death. However, the activation of cell death-inducing mechanisms may be activated not by fraction composition only, but also by the cell type exposed to particular fractions or other confounding variables like the set up laboratory parameters and locality of plants (49, 50, 51, 52). Additionally, hypotension may potentiate the translocation of nuclear factor-kappa β (NK-k β) and activator protein

I to the nucleus and accelerate cell death (53). Furthermore, accelerated cell death is attributable also to the release of adhesion factors, inflammatory cytokines and chemokines in response to reactive oxygen species caused by the hypotensive effect in the first place (54, 55). This overall contribution of reactive oxygen species in human cell culture affects the interaction and cytotoxicity readings of compounds with cell lines, which is known to activate the cascade of Bax and cytochrome c translocation and caspases; that results in apoptosis (56).

The activation of NK-k β is involved in eukaryotic cell death. The interplay of NK-k β activation with anti-oxidants present in plant fractions has the potential to reduce cell death. Therefore, the cytotoxicity results of some plant fractions (or compounds) may be incorrect, and thus, in the presence of cell proliferation, compounds being non-cytotoxic purely may be untrue. This was probably the case for *Baccharis drucunculifolia* and *Platycodon grandiflorum*, and for some fractions that show very low cytotoxicity percentages in human cell culture and are reported as non-cytotoxic or proliferative. The latter occurs because some anti-oxidants contain reactive oxygen species scavengers, that prevent cellular damage. However, as mentioned, some cell lines may be resistant to certain compound concentrations. Reactive oxygen species (hypoxic or hypotension) are dissolved within cells by scavenger or enzymes (called catalase and superoxide dismutase), that offer protection against reduced cytokines and plasma levels. In addition, they also preserve the anti-oxidant and anti-apoptotic protein content of cells. This offers conservation to read cytotoxicity readings in human cell culture probably, but obviously not entirely. However, it has been shown to induce pro-apoptotic signaling by keeping the ration of Bcl-2/Bax proteins in high proportions in cells, in addition to increasing the expression of CCP32, the cleavage of poly-(ADP-ribose)-polymerase (PARP) and the reduction in the fragmentation of DNA (57, 58). These events have been singled-out during the induction of apoptosis in HEp-2 cells (59).

Still, as noticed, reactive oxygen species is not the only variable impeding the interaction and cytotoxicity of compounds in human cell culture, and a lot of animous effort has been put in attaining completely reliable cell death results for infectious diseases for example, with genetic experiments and other unrelated tests for assaying the properties of compounds. It is expected that in the future, this need be eliminated in conjunction with performing tests on the properties of compounds, thus increasing the strength and efficiency of scientific results for pharmaceutical and research industry benefits.

CERAMIDE INTERFERENCES OF ANIMAL CELLS

Although it is known that ceramide forms an important component of animal cell membranes, its interference on cytotoxicity percentages in human cell culture has not really been reported in a precise way to the scientific public. This

interference may either enhance or diminish the interaction of different hydrophobic and hydrophilic compounds with different robust cell lines, since ceramide is essentially made up of a combination of sphingosine and fatty acid (60). Ceramide was discovered centuries ago, and its use as a synthesized compound or stimulus has commonly been reported, as shown by a previously reported example. Compounds that are hydrophobic usually enter human cell culture easily because they attract toward the inner components of the cell. These components are more concentrated than the exterior or tissue culture medium in which the cells bathe in. Based on the rate utilization of particular compounds, cells may experience distress, relaxation or an increased ability to proliferate. However, compound or plant fractionations often cause an alteration to the actual therapeutic value of fractions, resulting in the cytotoxicity of the actual constituents being unreliable. Therefore, ceramide includes another variable that needs to be eliminated for efficiency in cytotoxicity and interaction methodologies in human cell culture.

Recently, no work has been reported on the interference of ceramide on the interaction/cytotoxicity of compounds in human cell culture, apart from experiments in lipid biochemistry that report the peroxidation of lipid membranes as a result of reactive oxygen species present in plant material (as discussed). An advancement to these studies would be to include the role of ceramide in cell death induction and signalling in infected cells, which eventually triggers cell death proteins like *bax* and *bid*, resulting in cell execution and phagocytosis by macrophages. At present, there is limited information about ceramide interferences, and the lack thereof has probably forced the cytotoxicity results of compounds onto an inconceivable tangent.

Within membrane biology, there is an abundance of information about ceramide. However, in addition to this structural component, phosphosphingolipids called sphingomyelin are also available, and these assist the cell to carry out signal transduction events such as apoptosis *via* the receptor–ligand and mitochondrial mechanisms. It is well known that sphingomyelin is abundant in the outer leaflets of the plasma membranes and it constitutes 30% of all lipids. This phospholipid, which is restricted to the lumen of the cell, is formed when ceramide reacts with phosphatidylcholine to form sphingomyelin and diacylglycerol in the Golgi, rather than more restrictedly in the plasma membrane. It participates mostly in secretory and endocytic processes, and, as we know, plays a role in protecting the brain and in the transmission of nerve impulses (61). Studies in cancer biology have confirmed, in general, that ceramide functions as a second messenger in many cellular processes that include apoptosis, growth differentiation, suppression, transformation, proliferation, as well as the regulation of cell-to-cell and cell-to-substrate interactions and senescence (62). Therefore, when cells come in contact with a cytotoxic or non-cytotoxic substance, the substance first interacts with ceramide, which then generates a signal into the cell. This signal may allow the compound/s to be utilized either through membrane proteins, ion exchange

channels or the receptor-ligand mechanism (63). This step determines how the compound is used by the cell; and results in cell death, proliferation or both for some fractions. Utilization depends on whether the compounds are hydrophobic, hydrophilic or both.

The role of ceramide is therefore complex because on the one hand, studies report it as a synthesized chemical, while on the other hand, cellular biologists report it as a membrane component; however, both exist. It is therefore probable that ceramide interactions of compounds and cell membrane components have an influence on reported cell-to-compound interactions and related cytotoxicity fluctuations. However, beside the interference of ceramide in cell death events, several other influences on cytotoxicity results have been previously mentioned, and many others exist in the literature that publicly avails itself. The factors already mentioned therefore are affected by ceramide, and it is clear from this that elimination of those factors in screening pharmaceutically-important plants for their cytotoxicity and interaction with infected cell lines are probably and not entirely achievable, particularly in under-resourced laboratories and in developing country institutions. It is likely that understanding the attempts in eliminating ceramide interferences on compound cytotoxicity will provide improved infected cell culture results within the pharmaceutical industry in the long-term.

CHEMICAL MODIFICATIONS OF PLANT COMPOUNDS

Plants contain many different kinds of compounds and some plant species that belong to the same family have the same or similar substances – for example, the *Kniphofia* and *Bulbine* genera. However, even though some plant species have similar substances, some have analogues and are derived from others. For example, in general and in relation to this review, the *cis* and *trans* forms of compounds are chemically the same, but the positioning of hydrogen groups have an influence on their mode of action in cells (64). Similarly, during human cell culture studies, compounds that are analogues, may bring about different cellular responses and therefore indicate differences in the cytotoxicity levels between compound forms. However, many other cases have been reported. Although plant fractions or material contain many different secondary metabolites, in addition to their phenolic substances, substances that are similar or have the same structure may form macromolecules and adopt a 3-dimensional structure that could possibly interact with enzymes and the genetic code. Scientists believe that this feature is important for the future applications of compounds in medicinal industry, and that this feature requires further investigation for many therapeutically-important plant species.

Plant species and/or similar compounds exhibit different effects on cell lines for aqueous and organic fractions of different concentrations. Scientists found the antiproliferative effect of venom to be reduced due to heating over an extended period of time (36h) in HEp-2 and HeLa cell lines, and that further investigations

were required (65). Similarly, compounds present in fractions may induce cell death, but have a dilute cytotoxic effect compared to the compounds present in the crude plant material, because of the differences in fraction preparation. Furthermore, the frequently reported human cell culture incubation period of 37°C over an extended period of time requires the rotatory evaporation of ethanol from the organic fractions prior to fraction incubation, and this could alter the cytotoxic compounds present in the material (66). This could also contribute to irregular cytotoxicity percentages as well as proliferation for some fractions, instead of cell death and vice-versa.

CYTOTOXICITY AND CELL DEATH

Cytotoxicity and cell death assays are therapeutic areas of importance that have been used for decades to improve the wellbeing of public health, for example. However, from a medicinal perspective, cytotoxicity and cell death are unable to be correlated in any way because they report different scientific parameters. At present, attempts have been made to report additional scientific techniques that can confirm either assay, but very few attempts have been made to link the two concepts or techniques for analyzing the therapeutic importance of compounds and plant-derived fractions. A technique that has been used to confirm cell death, for example, is light microscopy, and for cytotoxicity, these techniques include the XTT and trypan-blue assays (as mentioned). Light microscopy is not necessarily accurate, because it is unable to differentiate cell death types precisely (*e.g.*, apoptosis from necrosis). The lack of reporting technique links have arisen, due to the irreproducible nature of the results from either assays to make proper correlations. For instance, a cytotoxic compound may induce cell death through a specific pathway and not another; or a non-cytotoxic compound (proliferative capability) may induce cell death or apoptosis through a specific pathway that is detected using specific markers in agarose gel electrophoresis (67).

Although not many investigations have been done on this, differences are likely to depend on the proportion and/or concentration of cytotoxic administered compounds in human cell culture. Furthermore, cases have been reported and discussed in this review, where human cells utilize compounds at higher concentrations for growth purposes, while at lower concentrations, they induce death and vice versa. This has been attributed to the genetic machinery differences between different cell lines, as well as the proportion of phytochemical substances between serial dilutions. Recent reports state that Polymerase Chain Reaction (PCR) is the most accurate and reliable way of detecting cell death (68); however, this technique is too expensive to perform in developing countries. Reverse-transcriptase PCR has been reported for transcribing genes that are important in the diagnoses of various ailments such as HIV/AIDs, cancer, tuberculosis, and many

others. As many scientists are performing cytotoxicity and cell death assays with different laboratory variations to protocols, it is likely to see the development and advancement of correlations and/or reporting irregular and/or regular results over the next couple of decades at least.

CONCLUSIONS AND PERSPECTIVE

In the context of this review, the cytotoxicity of a compound or death of human cell culture is influenced by many parameters that have an impact on the interaction of compounds with human cell lines. Thanks to the presence of several scientists and modern biotechnology, understanding the interaction of compounds with human cell culture may help engineer or re-engineer drugs that target specific pathways of cell death, particularly in infected or tumour cells. Common to molecular biology, although RT-PCR is the most advanced technique of analyzing cell death induction mechanisms induced by (cytotoxic) compounds, expressed/suppressed markers need to be correlated with cytotoxicity of administered compounds in comparison with different infected cell lines. Resource limitations are a major impending factor in developing countries, however, understanding important patterns between regulation of specific genes in human cell death pathways in relation to the cytotoxicity of compounds may help understand the interaction of those compounds with human cell culture in the search for therapeutic cures in the long-run.

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