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The Cancer Cell Thiolome – Is it Time for A Re-Evaluation of its Therapeutic Potential?

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ABSTRACT

This review discusses the role of various thiol constituents of the eukaryote cellular thiolome, particularly their role in the metabolism of tumour cells. The impact of the presence of a new unknown part of the thiolome is discussed which may have important ramifications in the search for new and more effective chemotherapeutic drugs to treat metastatic tumour cell growths.

Keywords

Cancer cells, Thiols, Proteins, Cysteine Thiolome.

General Introduction

In living organisms, low-molecular weight thiols are a class of small molecules featuring a carbon-bonded sulfhydryl group. They have long been known for their redox-relevant role in protecting against various endogenous and exogenous stresses. Thiols are crucial metabolically active compounds: they constitute the major metabolic and reactive cellular constituents responsible for many of the vital and important processes of life. These organosulphur compounds maintain intracellular redox homeostasis, function as reductive cofactors, and act as scavengers involved in the detoxification of alkylating agents, free radicals, and xenobiotics.

In the field of cancer research the role of thiols in in protection against xenobiotic and carcinogenic attack has been clearly established in radiosensitization and protection. Furthermore, recent trends have predominately illustrated the importance of thiol compounds in the role in the control of cellular redox levels and signal transduction in addition to of the control of oxidative stress. The latter causes injury to cells, inducing gene mutation, and is involved in carcinogenesis by influencing intracellular signal transduction and transcription factors directly or indirectly via antioxidants. Oxidative stress can be caused by the generation of reactive oxygen species (ROS), the overproduction of which seems to play a major role in the metabolism of cancer cells. There have been a large number of reviews published discussing this controversial topic [1-3]. Recently Domenicotti and Marengo [4] discussed the "state of the art" in the paradoxical role of oxidative stress in cancer illustrating the complexity of the subject.

Since thiols are one of the major cellular groups involved in the removal of harmful ROS, the implications for ROS regulation are highly significant for cancer therapy as it has been shown that some commonly used radio- and chemotherapeutic drugs do influence tumour outcome through ROS modulations often involving thiol metabolism. It has therefore become evident that the exploitation of the thiolome presents an area of high potential for the development of new and more effective chemotherapeutic drugs especially those that can be used to treat intractable metastatic cancer growths now responsible in some cases for greater than 90% of cancer-related deaths and for which no effective therapy is available. Metastatic spread, not primary tumour burden, is the leading cause of cancer death. It has therefore become vitally important to find new therapeutic approaches in order to tackle this problem. This review attempts to identify the various current areas of interest in the known main areas of tumour thiolomes which may be of relevance in the discovery and development of new anticancer drugs. Also, it suggests in the light of the author's recent findings, that, in order to achieve success in the future, new lines of approach in this area of research are necessary.

The known contents of the Cellular Thiolome

In the last few decades or so during the evolution of the so-called cellular "omes", (eg such as metabolomes, proteomes etc.,) the term **thiolome** is being increasingly been used as a broad description of the cellular thiol constituents. In terms of these constituents the identity and levels of the various types of low and high molecular weight thiols can vary widely.

Although many attempts have been made to identify and quantify the thiolome constituents of eukaryote cells this has proved a difficult task from an analytical point of view owing to the reactivity and ease of oxidation of various known cellular thiol groups. Thus, in order to effectively study these entities and prevent the formation of oxidation artefacts it has become necessary to use specific and stable blocking agents to produce detectable adducts. This has led to the development of a plethora of chromogenic, fluorescent and also radioactive labelling compounds for measuring and identifying cellular thiols which have revealed the complexity of the cellular thiolome. Broadly speaking, the cellular thiolome can be divided into two areas; those involving the thiols which are part of protein structures, sometimes referred to as the Cysteine Proteome, and those involving the low molecular weight thiols of the cell which can be described as the Low Molecular Weight Thiolome (LMWT); interest in the latter has been mainly focused on the ubiquitous cysteine containing tripeptide known as glutathione (GSH).

The Protein/Cysteine Thiolome

Cysteine is one of the most chemically reactive natural amino acids found in cells yet it is one of the least abundant of the amino acids, frequently found as a highly conserved residue within functional, regulatory, catalytic, or binding, sites in proteins.

In highlighting the relevance of cellular thiol groups in cell metabolism the important role of cysteine containing proteins has been well documented over time. It is often assumed that the bulk of the thiol containing material in eukaryote cells resides in the cysteine residues present in their protein moieties.

In an early review of the thiol contents of different cellular proteins and enzymes by Guzman Barron [5] it was noted that the behaviour of these thiol groups towards the various detection reagents differed with the protein studied. Some thiol groups were described as *freely reacting* while others were described as *sluggish* were only detected with the more powerful reagents. However, other thiols seemingly concealed within the protein matrices were only revealed on total denaturation of the protein with strong chaotropic reagents such as high concentrations of urea or guanidinium hydrochloride. He called them *masked* -SH groups; in many cases he found that they amounted to approximately 50% of the measurable protein thiol although he concluded that they were not, in general, all connected with enzyme activity. At the time it was clear that protein thiol groups, assumed to be mainly cysteine in nature, played vital roles in the maintenance of cell division and homeostasis.

In later work a number of investigators, in analysing the published data on the cysteine and half-cysteine content of intracellular proteins, found that most of them have a low cysteine content. The mean cysteine content of many intracellular proteins amounted to only 1.6%, which is only about half the predicted value; extracellular proteins by contrast have been found to have a value of 4.1%) [6]. Thus, it was concluded that intracellular proteins have a low, but generally nonzero, cysteine content. This is surprising in view of the many established important metabolic functions of some cellular proteins, which need intact thiols for activity. Over the years many investigators have clearly established that, after extraction of the cellular glutathione (the main low molecular weight thiol reported in eukaryote cells), the protein fraction/precipitate contains up to 75-80% of the measurable cellular thiol. As a result of his earlier thiolome studies this has suggested to the author that thiol containing moieties other than cysteine could be present in the denatured protein matrices. As a result of more recent thiolome research Go et al. [7] stated that the "cysteine (Cys) proteome" is a major component of the adaptive interphase between the genome and the exposome and that the thiol moiety of cysteine undergoes a range of biological modifications enabling biological switching of structure and reactivity. Poole [8] reviewed the importance of cysteine containing moieties, mainly being present in the proteins, in redox biology and chemistry. He concluded that modifications of cysteinyl residues can impart or regulate molecular functions important to cellular processes, including signal transduction.

Wu et al. [9] used the term **Cysteinome** based on the amino acid cysteine being the main thiol component found in the structure of proteins. They concluded that redox sensing, signaling, adaptation and associated disorders are inextricably linked to site-specific covalent modifications of the cysteinyl proteome. Although many important eukaryotic cellular enzymes require intact thiol groups for their activity, in many cases, such as the nucleic acid polymerases, their exact mode of action seems not to be clearly understood. However, the following protein thiolomes have been extensively researched.

a) The Iron-Sulphur Proteins

One of the most interesting parts of the Cysteinome are the iron-sulphur proteins. In 1967 the cysteine containing iron-sulphur proteins were discovered and they have since been described as "ubiquitous biological redox reagents". Extensive reviews on these proteins have been published, for example by Orme-Johnson and more recently by Rouault [10,11]. In many cell types they undoubtably form a vital part of the cellular thiolome though in the case of eukaryote cells it doesn't seem clear exactly what roles they play in the various cell compartments.

Very recently a number of reviews have appeared on the role of these iron-sulphur proteins in cancer metabolism. For example, Daher et al. postulated that cysteine depletion could be a key action to challenge cancer cells to ferroptotic cell death [12], Further studies have shown that iron-sulphur clusters are closely related to the mechanisms of multiple cell death modalities. In

addition, numerous previous studies have demonstrated that iron-sulphur clusters play an important role in the development and treatment of cancer. This subject has been recently reviewed by James et al. [13] and also Zhang et al. [14]. The latter attributed perturbation of iron-sulphur clusters as a key factor of regulated cell death in cancer.

b) Zinc finger proteins

The ubiquitous Cysteine-Zinc finger proteins form one of the largest families of transcription factors in human genetics, via their conserved zinc finger motifs (see https//en.wikapedia.org/wiki/Zinc finger). They function in many biological processes including development, differentiation, metabolism and apoptosis. Recent studies [15] have reported that they are closely associated with different stages of cancer development. One of the hallmarks of cancer is altered signal transduction cascades and these authors believe that an understanding of the changes in these pathways is essential for targeted cancer therapy. They discuss examples of zinc finger proteins being involved in the development and progression of several types of cancer, providing possible new insights into cancer therapy possibilities.

c) Redoxins -Thioredoxins, Peroxiredoxins and Glutaredoxins

Another important part of the cysteine thiolome is the class of redox proteins known as Redoxins.

These redox controlling thiol containing proteins have long been linked to cancer development and metabolism [16]. Since then, because oxidative stress has been established feature of many cancers [17], many studies on these proteins have been focussed on this aspect of redoxin metabolism in tumour cells. For example, in an extensive review Harris, I.S. and 25 co-authors revealed the possible roles of the glutathione and thioredoxin antioxidant pathways in synergizing and driving cancer initiation and progression [18].

In a further development Bhatia et al. [19] pointed out the possibilities of improving the breast cancer patient outcomes by targeting the thioredoxin (Trx) system with auranofin and other specific inhibitors to inhibit cancer invasion and migration. Later Hopkins and Newman reviewed the accumulating evidence that these proteins are the gatekeepers of transcriptional oxidative stress response [20].

In an interesting review Karlenius and Tonissen discussed 8 possible roles of thioredoxin in cancer metabolism [21]. They concluded that thioredoxin protein levels are elevated in many human primary cancers and this high expression is associated with aggressive tumor growth and inhibited apoptosis, as well as decreased patient survival and resistance to anti-cancer treatments.

In a later review Ghareeb and Metanis point out that, in view of the wide range of cellular functions including redox control played by the thioredoxin system and that this system is overexpressed in many cancers, makes it s promising target for cancer drug development [22]. They suggested that more efforts should be put into the development of protein/peptide-based inhibitors against thioredoxin reductase and/or thioredoxin itself. Additionally, a stimulating review article has been published very recently discussing the roles of thioredoxin-interacting protein (TXNIP) with thioredoxins in cancer. The authors describe this system as a fine balance between redox, metabolic and immunological tumour control [23]. They conclude that an improved understanding of the functions and mechanisms of TXNIP may enhance its suitability as a therapeutic target.

d) Glutathione S-Transferases (GST)

This important ubiquitous supergene family of enzymes represents a major group of complex detoxification enzymes present in multiple forms in all eukaryote species. An enormous amount of research has been carried on their structure and function, which has been reviewed in depth by Hayes and Pulford [24]. 766 references on this work have been quoted in their review ranging from their mapped sequences to their roles in cellular metabolism in oxidative stress to control of gene expression.

They are a family of enzymes that catalyze a number of distinct glutathione-dependant reactions; they all possess the ability to conjugate glutathione with compounds containing an electrophilic centre and thus form an important part of protein thiolome. The fact that these enzymes contain a number of different catalytic groups which include a cysteine residue makes them an important part of the protein thiolome. Individual GST genes are each regulated in a distinct fashion and each encodes a protein with unique catalytic activity.

e) Metallothioneins

The Metallothioneins are a family of cysteine rich low molecular weight proteins with molecular weights ranging from 500-1400. They have the capacity to bind physiological and xenobiotic heavy metals through their cysteine residues. Originally discovered by Vallee and Margoshe in 1957 in equine renal cortex they have been found to be expressed in a vast range of taxonomic groups; in the human body they are synthesised mainly in the kidney and liver.

They have been mooted to have potential roles in human carcinogenesis. Many studies have shown that there is increased expression of metallothioneins in a wide variety of human cancers; evidence suggests that greater metallothionein expression may cause resistance to chemotherapy.

In addition to generally protecting against metal toxicity the biosynthesis of metallothionein appears to increase several-fold during periods of oxidative stress to shield the cells against cytotoxicity and DNA damage (see Wikipedia for further details).

f) Rhodanese

Rhodanese is a ubiquitous enzyme found in both animal and plant kingdoms. It is an important mitochondrial enzyme containing a

vital active thiol grouping that that detoxifies cyanide by converting it to relatively harmless thiocyanate. Some investigators have studied the role of this enzyme in cancer cell metabolism and carcinogenesis but there have been no indications of its potential for therapeutic use seem to have emerged - except perhaps in its role in the detoxification of the anticancer agent amygdalin. Cancer cells are thought to have more β-glucosidase and less of the liver enzyme rhodanese, which converts cyanide to relatively harmless compound thiocyanate. Thus, the cancer cells are more susceptible to the effect of amygdalin than the healthy cells.

g) SNARE proteins - N-ethylmaleimide-sensitive factor attachment protein receptors

First described in the 1980's SNARE proteins (Soluble N-ethylmaleimide-sensitive factor attachment protein receptors) are a group of proteins containing metabolically active thiol groups that play a crucial role in the final step of membrane fusion in eukaryotic cells. They form a complex that mediates the fusion of vesicles with their target membranes and are also involved in the regulation of subcellular trafficking of ion channels. They are now generally accepted to be the major players in the final stage of the docking and subsequent fusion of diverse vesicle-mediated transport events [25]. The role of these proteins in tumorigenesis and their potential as targets for anti-cancer therapeutics has been discussed in depth by Meng and Wang [26].

The Low Molecular Weight Thiolome

a) General low molecular weight thiol content of cells

In this class there are a number of well-known low molecular weight thiols that are involved in important cellular metabolic processes, such as CoenzymeA in mitochondrial metabolism. A table showing the known cellular low molecular weight thiols is given in supplementary information. However, during the in-depth quantitative analysis of thiols in whole eukaryote cells, most of these low molecular weight thiols are barely detectable, probably because many are transient in nature.

The only low molecular weight thiol currently accepted to be present in any quantity is glutathione (L-γ-glutamyl-L-cysteinyl-glycine (GSH,) which discovered by Sir Fredrick Gowland Hopkins in the early 20th century. As a result of much intensive research since then it is now widely accepted that GSH is the major low molecular weight present in most cell types studied. It is often quoted to be present at concentrations of 5 millimolar or more. It has been estimated that GSH accounts for more than 90% of the total nonprotein sulphur of the cell [27]. Some its components such as cysteine and cysteinylglycine and traces of cysteinylglycerate) can also be detected, but in lower concentrations [28]. Sometimes, in various pathological conditions, homocysteine is found, but it is mainly detected extracellularly. GSH has been shown to play an important role in a multitude of cellular processes, including cell differentiation, proliferation, and apoptosis. There are many reviews on this subject, recent ones are now thankfully freely available on the internet.

b) GSH and Cancer

As a result of much recent research work it is now accepted that GSH is an important factor in the control of oxidative stress in cells. While GSH deficiency, or a decrease in the GSH/glutathione disulphide (GSSG) ratio, leads to an increased susceptibility to oxidative stress implicated in the progression of cancer, elevated GSH levels can increase the antioxidant capacity. Resistance to oxidative stress is observed in many cancer cells. Many reviews on this subject have now been published, for some examples see Harris et al. [18], Giles [29], Acharyah et al. [30], and Castaldo et al. [31].

It has been shown that malignant cells have significantly higher GSH contents than non-tumourigenic cells, and that GSH depletion impairs the viability of tumour cells and reduces their metastatic spread when transplanted *in vivo* [32]. Early on Arrick et al. [33] showed that inhibition of glutathione synthesis augments lysis of murine tumour cells by sulfhydryl-reactive antineoplastics. The role of GSH in cancer metabolism has been discussed in a great number of different reviews. To quote a few early examples; Balendiran et al. [34] discussed the role of GSH in cancer biochemistry, also Estrela et al. [35] have reviewed the role of GSH in cancer biology and therapy. In a later review Traverso et al. [36] considered the evidence for the role of GSH in cancer progression and chemoresistance. Bansal and Celeste Simon considered the significant roles that GSH and GSH-related moieties might play in tumour initiation, progression, and drug resistance [37]. They concluded that targeting GSH synthesis/utilization represents a potential means of rendering tumour cells more susceptible to different treatment options such as chemotherapy and radiotherapy. Very recently in a comprehensive review Kennedy et al. [38] summarised research on the role of GSH in cancer from mechanisms to therapies. They found that several novel therapies have been developed to target the GSH antioxidant system in tumours as a means for increased response and decreased drug resistance.

In summary, there has been considerable evidence presented to show that GSH plays a major role in cancer genesis and metabolism. A common characteristic of many cancer cells is they suffer from oxidative stress which can be controlled by GSH. Although some success has been obtained in animal tumour models it remains to be seen whether there have been many successful clinical trials on the drugs developed as result of GSH research. However, this may be taking place in some multinational drug company or other. In this field of thiol research there is still currently much hope that a better understanding of GSH metabolism will lead the development of mechanism-based GSH inhibitors, which can then be combinatorically used with other drugs to effectively limit tumour growth.

c) Recently Discovered Unknown Low Molecular Weight Thiols

During many years of thiology research the author has been intensely interested role of thiols in cancer cell metabolism

with the aim of finding a weakness that might be exploited to improve cancer therapy. His early research on tumour thiols in the 1960's centred collectively on what has now become known as the cellular thiolome. At the time low molecular weight thiols in present in tumour cells were known to play important roles in radiosensitization and protection; also via their reaction with chemotherapy drugs such as alkylating agents in clinical use at the time [39]. In the 1960's it was well known that there were significant quantities of unknown low molecular weight thiols present in eukaryote cells, but they had been found to be difficult to isolate and identify. None were identified. However, in the course of some early studies by the author on nuclei isolated from various rat tissues and a rat tumour cell line new low molecular weight thiols were detected. Adducts of these thiols were isolated but not identified. Owing to a career change in the 1980's this work was not published until much later [40]. However as a result of work done in the mid 1970's, the results of these studies were confirmed and extended [41]. At this point, due to a career change, this research was suspended for 20 years and then taken up in private laboratories. In continuing this work over the last 10 years or so the author's work has been exclusively involved in the analysis of the thiolome of some human prostate cell lines, particularly on a metastatic cell line known as LNCaP.

Initially the thiolome of these cells was accurately quantified using the well-known thiol reagent known as the Ellman reagent. These experiments revealed that the thiol levels of protein in these cells were much lower than expected and that unknown low molecular weight thiols were present [42]. Later experiments using a coloured aromatic mercurial label to visualize these thiols confirmed these results and revealed the nature of the latter which were deemed to be **"Conthiols"** [43]. LS-MS studies on this adduct revealed that only one major species of this thiol could be detected. It was shown to be present in concentrations four times greater than the GSH present in these cells.

At this point in time the following information is available on the nature of this thiol, which is partially hidden in the cellular protein matrices has emerged; it is as follows:-

- Conthiols can only be fully detected after dissolving cellular material in high concentrations of strong chaotropic reagents which open up the protein matrices.
- They are not covalently bound to protein.
- They generally constitute 56.5 to 61.4 percent of the total cell thiol content -nearly four times the concentration of GSH present in the human prostate cell lines studied.
- At present they can only be isolated in the form of a suitable stable adduct such as those formed from organomercurial compounds.
- They do not contain any GSH, amino acids, ribose or deoxyribose or nucleic acid bases.
- They do not contain phosphate or amino groups (primary amines) but are strongly adsorbed on both anion and cation exchange columns indicating the presence of a zwitterionic group which could be a strong chelating agent.
- Based on LC-MS analysis of a mercurial adduct there appears to be one major component which has a possible molecular weight of 467
- Once released from their protein "scaffolding", these thiols are unstable and are barely detectable in the cell sap.
- When released from the mercurial adduct label they break down to give a thiol containing moiety and a non-thiol compound neither of which have been fully identified. However, a thiol containing portion has been shown to consist of a polymer of five repeating units each with a mass of 58 daltons; possibly a nitrogen containing moiety such as N-CO2 [44].

This long and varied work on the composition of cellular thiols which led to the discovery of the Conthiols was carried out by the author intermittently over some 60 years has been recently reviewed [45].

Conclusions and Discussion

Much research has been done on the metabolism of various components of the tumour thiolome that have been discovered but, due to the instability of compounds containing the thiol group, this is a field of biochemistry research in which it is difficult to achieve meaningful, reproducible and reliable results. This has led to the publication of many, sometimes confusing, scenarios of thiol metabolism appearing in the published literature.

In the last few years there has been a spate of papers and reviews on various aspects of the cancer cell thiolome clearly pointing out the complexity of this area of cancer research. Most have been concentrating on the role of redox control and oxidative stress in tumours together with cell signaling aspects. Many investigators have been looking for weaknesses, which might be exploited for new anticancer drug development. The author's early conviction that GSH is not the major low molecular weight thiol, has led him to continue his thiol studies late into retirement. The last ten years have been rewarded by being able to show convincingly that unknown and unstable, very reactive low molecular weight thiols, deemed to be Conthiols, are present in human prostate tumour cell lines. It is important to note that, the light of his earlier studies on isolated nuclei and nucleoli, it became apparent that Conthiols are almost certainly involved with both the RNA and DNA polymerases, many of which have been reported to contain up to 4 cysteines per mole of enzyme in disulphide form. The synthetic activity of these enzymes is highly dependent on intact thiol groups and so it would be interesting if Conthiols could be shown to be present in isolated forms of these enzymes. Because they are present in such high quantities Conthiols must play important roles in the control of cellular redox and oxidative stress – possibly more than any other thiol in the cell? Is it the missing piece in the "jigsaw" of the tumour thiolome?

Another suggestion by the author is that Conthiols may be the result of an evolutionary biochemical development from some highly reducing antioxidant mercaptohistidines found in nature. For example, Ovothiols which are biosynthesized by many marine

invertebrates, microalgae and bacteria (for recent review of this field see Brancaccio et al. [46]. Also, the thiolhistidine called Ergothionine which is made in relatively few organisms such as bacteria and certain fungi [47].

The fact that Conthiols are hidden/buried with the matrices of some proteins probably means that they do not act as signalling messengers or general controllers of cellular redox as do glutathione and other simple reducing sulphur moieties, such as sulphides, sulphones (S^0), persulphides, or hydrogen sulphide, all of which have been shown to be so important in cell signaling.

The structure on the Conthiol molecule will be unlikely to emerge until a means can be found to stabilize the thiol after release the free thiol from its label. Attempts to identify the molecule released from the mercurial label with standard available technologies have been thwarted by the instability of the isolated thiol when released from the label but some clues have emerged [44]. Until stabilization can be achieved future biochemical studies will be difficult without the use of radioisotopes such as ^{35}S for which special facilities are required but these are not available to him at present [35]. Finally, in the author's view there is no doubt that further investigations on the tumour thiolome will provide valuable information which will have great potential for the development of new drugs for cancer therapy, particularly in the light of the new, rapidly evolving AI platforms which can evaluate possible new protein/low molecular weight compound interactions.

Knowledge of the nature of the entrapment of Conthiols in protein matrices will surely be a vital factor for the design of new cancer therapeutic drugs. In this respect the use of artificial intelligence (AI) techniques could be used to speed up drug development and drug companies are beginning to think that the use of AI will lead to faster drug development (see recent Nature editorial *https://doi.org/10.1038/d41586-023-03172-6*). However, it is important to keep in mind that existing AI platforms are only valuable if computers are given the right data [48]!!.

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Supplementary Information

Formula weights of low molecular weight thiols commonly found in eukaryote cells (in g·mol−1)

Hydrogen Sulphide - 34.08 (Present in trace amounts, involved with signalling processes).

Cysteine - 121.16 (present mainly in protein structures).

Homocysteine - 135.18 (mainly extra-cellar, found in some pathological conditions).

α-Lipoic acid (reduced) - 206.32 (Naturally occurring lipoic acid is always covalently bound to macromolecules).

Pantetheine - 278.379 (intermediate in the production of Coenzyme A).

Glutathione - 307.32 (assumed to be major non protein thiol in cells).

Coenzyme A - 767.535 (present in trace amounts, can scarcely be detected in cell extracts).

Some other possible low molecular weight thiols not normally found or synthesised in terrestrial eukaryote cells.

Ovothiols are sulphur containing natural products biosynthesized by marine invertebrates, microalgae and bacteria. An example is **Ovothiol A** - 201 (N1-methyl-4-mercaptohistidine) a highly reducing antioxidant mercaptohistidine, which accumulates to very high levels in the eggs of certain marine invertebrates.

Ergothionine – 229 Made in relatively few organisms such as bacteria and certain fungi.