Effect of major tumor metabolites on the release of doxorubicin from Doxil – implications for precision nanomedicine.

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Graphical Abstract:

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Abstract:

Our previous studies demonstrate that ammonia induces doxorubicin release from Doxil[®] in a concentration-dependent manner. Because ammonia that results from glutaminolysis is continuously generated in tumors at high enough concentration to induce doxorubicin release of Doxil in tumors, this may explain why doxorubicin release in interstitial tumor fluids is much faster and higher than in the plasma, in which release is minimal. This unique activity of tumor ammonia may explain, at least in part, the therapeutic efficacy of Doxil, which in practice does not release doxorubicin in animal and human plasma. Our current study aims to evaluate if tumor-specific metabolites other than ammonia, such as lactate and pyruvate, are also involved in doxorubicin release from Doxil. Also, we studied levels of ammonia in other mouse organs. Our data shows that these other metabolites do not affect doxorubicin release. Furthermore, using the Metabolic gEne RApid Visualizer database (MERAV), we computationally explored the relationships of glutaminase (GLS) 1 and 2, glutamate dehydrogenase (GLUD)1 and 2, as well as glutamine transporters regulating the glutaminolysis levels found in different cancers. These glutaminolysis levels could not be achieved without the upregulation of glutamine transporters. Indeed, our queries to MERAV showed that SLC38A1, SLC38A2, and especially SLC38A6, are up-regulated in cancerous tissues. We discuss how the information on the upregulation of enzymes related to glutaminolysis could be used for “precision medicine” to determine if Doxil is an appropriate choice for a specific cancer patient. Our computational exploration shows that glutaminolysis is heightened in some cancerous tissues as compared to their normal counterparts, but not in all cases. It would be possible and perhaps advantageous to test individual patient tissues to determine glutaminolysis, and therefore likely, ammonium levels in cancerous tissues, and to use this to ascertain if Doxil would be a good treatment choice.

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Rationale and purpose:

Recent emphasis in nanomedicine has been given to stimulus-responsive release of drug from nanoparticles, not as much attention has been paid to the mechanisms of nanomedicine release already in the clinic, which were not designed with a specific stimulus release mechanism. Doxil shows almost no release in human plasma but does release near the tumor, which is critical to its clinical success in patient treatment. Our previous article explored this release mechanism, which we determined to be controlled by the ammonia present in tumor interstitial fluid. However, this discovery could be further explored and broadened to look at other potential sources of release stimulus for passive release nanomedicines. This paper looks at other potential mechanisms, and how they could be applied for more personalized medicine.

Introduction:

Due to the significant differences in bioenergetic and biosynthetic requirements between cancer cells and normal cells, most cancer cells use markedly different metabolic machinery than normal cells, as seen in Figure 1. The Warburg Effect (Heiden and Vander, 2018) describes the changes in glycolysis being aerobic in cancerous cells. These were the first changes to be observed and characterized. An outcome of these changes is that much of the pyruvate generated via the glycolytic pathway is converted to lactic acid, rather than being used to produce acetyl-CoA and ultimately, the citrate which enters the citric acid cycle. In order to compensate for the resulting changes in the production of acetyl-CoA and the citric acid cycle, cancer cells often increase glutamine (the most abundant amino acid in plasma) metabolism,

Figure 1: Normal cell and cancer cell glucose metabolism. (figure from Erickson, 2010, with permission). This comparison shows the importance of glutaminolysis in cancer cell metabolism.

referred to as glutaminolysis (Koglin et. Al, 2011, Moreadith and Lehninger, 1984), which is caused by an elevation of the activity of the enzyme glutaminase (Erickson and Cerione 2010)

This upregulation of glutaminolysis in cancer is regulated by C-Myc, HIF-1 and p53, among others., as seen in Figure 2. (Chen and Russo; Wise and Thompson) One of the primary by-products of glutaminolysis is ammonia, which

builds up in cancerous tissue at much higher concentrations than in healthy tissue. (Eng et al.; Heiden et al.) Initially, ammonia was seen as primarily a waste product created by tumor cells, but more and more, it is being recognized as intrinsic to the metabolic rewiring common to cancer cells. For instance, new research shows that ammonia replaces tradition nitrogen donors for glutamine synthesis in cancer cells (Gouirand et al.).

Figure 2: Upregulation of glutaminolysis in cancer cells. (Figure from Vermeersch and Styczynski, with permission)

The role of ammonia in cancer cell metabolism has gained such recognition that in 2017, Science published an article by Spinelli et al., demonstrating that ammonia can be used in cancer cells by glutamate dehydrogenase to convert α -keto glutarate to glutamate, which can then be used in the synthesis of proline and aspartate, and showing ammonia to be a key bi-synthetic metabolite for cancer cells (Spinelli et al.). Additionally, our research shows that high ammonia concentrations in tumor tissue trigger doxorubicin release from the liposome in the anti-cancer drug Doxil® (Silverman and Barenholz). As previously demonstrated and explained, the level and rate of release from nano-liposomes is significantly faster than of large liposomes which point to the importance of the fact that Doxil are nano-liposomes (Silverman and Barenholz 2015).

Additional evidence for the importance of glutaminolysis in cancer cell metabolism is the increase of glutamine transporters in cancer cells. Indeed, it has been shown that shRNA knock-down of glutamine transporter ASCT2 inhibits proliferation in some cancer types. (van Geldermalsen et al.)

We have previously demonstrated that ammonia is continuously generated in tumors of mice

and can stay at levels as high as 5 mM, while ammonia levels in blood plasma are negligible, and that Doxil shows correspondingly high release rates in tumor tissue while having little to no release in the blood (Silverman and Barenholz). Additionally, in vitro experiments show that the addition of ammonia to a saline buffer solution induces the release of doxorubicin from Doxil present in this buffer, and the released doxorubicin kills tumor cells in culture with the same efficiency as free doxorubicin (Silverman and Barenholz).

However, there are other pathways and corresponding byproducts which are much more prominent in cancer cells than healthy tissue, which might also contribute to the release of doxorubicin.

As detailed in the 1964 article by Gullino et al., the interstitial fluid of cancer cells has been found to contain much lower concentrations of glucose than healthy cells, and corresponding higher concentrations of lactic acid and lactate, because of the aerobic glycolysis process cancer cells use to metabolize glucose, and the higher glucose requirements of cancer cells, sometimes called 'glucose addiction' (Gullino et al.). Aerobic glycolysis has also shown to prevent pyruvate from entering the TCA cycle

in glycolysis, causing a higher concentration of pyruvate in the interstitial fluid (Carins, 2011). Additionally, other studies have shown that glutaminolysis produces alanine as a byproduct, in addition to lactate and ammonia (patent), and that under certain conditions, cancer cells secrete glutamate rather than using it for biosynthesis (Tardito et al.). Other studies have shown that the increased ROS found in cancer cells leads to an accumulation of hydrogen peroxide (Lopez-Lazaro, 2007).

Tumor interstitial fluid (TIF) composition is always in flux due to the balance between its formation by trans-capillary filtration and the clearance via the lymphatic system. In 1964, Gullino et al., used rat tumor models to compare TIF, plasma, and the interstitial fluid of normal subcutaneous tissue. (Gullino et al.) These authors showed that the TIF enters and leaves the tumor and is characterized by much higher lactic acid and much lower glucose levels than in blood. The level of free amino acids was also higher in TIF. While extensive research has been done since the end of the 19th century on the tumor micro-environment, tumor interstitial fluid (TIF) studies are surprisingly few. In a recent comprehensive review, TIF is described to be hypoxic and acidic compared with plasma and subcutaneous interstitial fluid (Haslene-Hox et al.).

Experimental Design:

Experiments were divided into three stages; (1) further release testing of Doxil in buffers containing ammonia and physiologically relevant ammonia salt counter ions, (2) testing of non-cancerous mouse organs for native levels of ammonia in their interstitial tissue fluids, (3) and using the MERAV database to correlate gene expression of genes involved in glutaminolysis with organs having increased levels of ammonia, or with certain types of cancer that would then be candidates for treatment with Doxil.

Materials and Methods:

Liposomes were prepared and the release was tested as described in our previous paper. (Silverman and Barenholz) Briefly, Doxil, Caelyx, or a generic equivalent, DOX-NP (prepared by Lipocure LTD, Jerusalem Israel and distributed by Avanti polar Lipids (Albaster AL, USA), were used. For details on Caelyx (Doxil) see Janssen Products websites, and for

more details on DOX-NP see the Avanti Polar Lipids website from 2016).

Animal experiments were performed as per the joint ethics committee (IACUC) of the Hebrew University and Hadassah Medical Center approved the study protocol under Permit Number: MD 20213 02. The Hebrew University is an AAALAC international accredited institute. Mice were maintained under standard conditions, $23 \pm 1^\circ\text{C}$, 12-hour light cycle (7 a.m.–7 p.m.) with ad libitum access to food and drink. The cation exchanger Dowex™ 50WK-400 (Sigma) was used as a doxorubicin sink to prevent any re-encapsulation of the released doxorubicin back into the liposomes due to residual liposomal transmembrane ammonium and pH gradients. In our current release experiment, we used this Dowex in excess; 30 mg of Dowex to 40 μg of total doxorubicin diluted in 1 mL of histidine-saline buffer. Under such conditions, the Dowex serve as an efficient sink for the released doxorubicin (see below). We determined that under these conditions, the ammonium did not interfere with doxorubicin binding to the Dowex-50. Samples of Caelyx or DOX-NP liposome product were diluted in buffered saline with varying concentrations of ammonium salt ranging from 0 to 50 mM, or in buffered saline alone. The buffered ammonium salt solutions were composed of commercial sterile pyrogen-free saline, 0.9% NaCl, 10 mM histidine at pH 7.4 and the appropriate concentration of ammonium salt. The solution was adjusted to the specific pH of the experiment using HCl and NaOH. Buffered saline consisted of 0.9% NaCl and 10 mM histidine pH 7.4 and adjusted to match the pH of the ammonium solution for which it was a control. Commercial PLD (Doxil or Caelyx) or DOX-NP samples contain a doxorubicin concentration of 2 mg/mL a 16-mg/mL total lipid concentration.

After dilution in the release medium, doxorubicin concentration was 40 $\mu\text{g}/\text{mL}$ and lipid concentration at 320 $\mu\text{g}/\text{mL}$. Samples were incubated at 37°C with shaking at 50 rpm for the specified time durations. Samples were removed from incubation at the given time points and assayed using the BioTek Synergy™ 4 Hybrid Microplate Reader for doxorubicin concentration from the absorbance at wavelength 490 nm. The percentage of doxorubicin released was calculated from the ratio between

the amount of liposome-encapsulated doxorubicin in the sample at any given time point and encapsulated doxorubicin at time T_0 .

Determination of ammonium/ammonia of healthy mice organs

In order to determine ammonium/ammonia concentration in healthy mice organ interstitial fluids we followed the process used to determine ammonium/ammonia levels in the tumors of tumor-bearing mice (Silverman and Barenholz 2015). However, the ammonium/ammonia determination was done by ion-chromatography, on Metrosep C6 250 $\times$ 4.0 mm (Metrohm 6.1051.430) column and elution was with a 2.5-mM nitric acid, conductivity detector. Six mice were used for the analysis of each of the organs presented in Figure 3.

The MERAV database was used as described by Shaul et al. Details can also be found on the database website: <http://merav.wi.mit.edu>. Briefly, cancerous or non-cancerous cells were selected, and the gene of interest was inputted into the database, which returned expression levels of the chosen gene in a variety of cell types. Plots were created by the MERAV database, as described in A. Shaul, Y.D., Yuan, B., Thiru, P., Nutter-Upham, A., McCallum, S., Lanzkron, C., Bell, G.W., Sabatini, D.M. (2015) MERAV: a tool for comparing gene expression across human tissues and cell types. *Nucleic Acids Res.* 44 (560-566), where the statistical methods are laid out in more detail.

Figure 3: Ammonium ion concentrations in mouse organs. $N=6$. Error bars represent standard deviation.

Building on our finding that ammonia induces doxorubicin release from liposomes, further experiments were done with other substances relevant to tumor interstitial fluids. In these evaluations, we tested lactate, pyruvate, and carbonate, as well as hydrogen peroxide, and found that none of these increase release rates in vitro. Hydrogen peroxide appears to inhibit the release slightly.

There was no further analysis beyond what is done by the MERAV database itself. The database was simply queried and then asked to provide boxplots of the data it produced. To our knowledge, while anyone can access this database and could query these specific genes and tissues, no one else has published these queries to elucidate glutaminolysis and ammonia levels in healthy and cancerous tissues.

Results:

Ammonium/ammonia concentration of organs harvested from healthy mice was determined using ion-chromatography with conductivity detection. It showed minimal ammonia in the lung, spleen, heart, and kidneys but with somewhat higher brain and liver levels.

Figure 4: Release of doxorubicin from liposome in solutions of ammonia with various counterions.

Additionally, we used the web-based microarray analysis tool MERAV to examine gene expression of glutaminase (GLS) 1 and 2, glutamate dehydrogenase (GLUD) 1, and the primary genes coding for the enzymes used in glutaminolysis, in both healthy and cancerous tissues, and found that while GLUD1 and GLUD2 are expressed in both healthy and cancerous tissue, in several organs, there is virtually no expression of the genes GLS and GLS2 in healthy tissue, and significant expression in cancerous cell lines. This is supported by Spinelli et al., who state in their article that mRNA for two other glutamine catabolism enzymes, glutamine synthetase (GS) and NADP-specific glutamate dehydrogenase (GDH1), is upregulated in many cancer types (Spinelli et al.).

Discussion:

GLS codes for K-type mitochondrial glutaminase, which catabolizes glutamine to glutamate and ammonia are found mainly in the brain and kidneys. This explains our findings of higher ammonia levels in brain tissue than in other mouse tissues. GLS2 has a similar function to GLS1 and is concentrated in the liver, although also present in other tissues, which correlates with our findings that ammonia levels are highest in the liver. While this graph gives an overall picture, more detail is seen when the organ data are separated (Supporting information, Figures S1, S2, S3, and S4)

Figure 5: MERAV graphs of gene expression of GLS and GLS2. Red represents normal tissue while blue represents cancer cell lines. While this graph gives an overall picture, more detail can be seen when the organ data are separated, as seen in supplementary figures S1, S2, S3, and S4.

GLS and GLS2 are more highly expressed in breast cancer and lung cancer than in healthy breast and lung tissue, respectively, and much higher in cancerous kidney tissue than a healthy kidney, but both variants of GLS are expressed more highly in healthy tissue in the brain and liver.

GLUD1 is more highly expressed in healthy tissue in all the organs tested. GLUD2 is higher or about equally expressed in all the tissues except the brain where it is more highly expressed in cancerous tissue, the only gene in the four genes examined where this is true.

The high expression of glutaminolysis genes in brain is explained by the fact that glutamate is one of the primary neurotransmitters, and it is created by breaking down glutamine using glutaminolysis. Glutamine can be broken down by one of two glutaminases which are used in the brain and are coded for by GLS and GLS2 (Márquez et al.).

GLS2 is in part regulated by p53, which is downregulated in cancer tissue. Therefore, logically GLS2 is expressed more highly in healthy liver tissue than in liver cancer, and indeed this was experimentally found by Suzuki et al., as published in *PNAS*, 2010.

GLUD1 and 2 are expressed in healthy cells' mitochondria and therefore do not correlate with cancerous tissue. GLUD1 is a housekeeping gene that is expressed in the mitochondria of all cells. GLUD2, however, is primarily expressed in nerve tissue. In most tissues it is expressed at a basal level whether or not the tissue is cancerous, but in brain cells, it seems that

GLUD2 expression greatly increased when glucose is low and the cell switches from glycolysis to glutaminolysis, a change that is seen only in cancer cells. This has been demonstrated experimentally in SF188 glioblastoma cells by Yang et al (Cancer Research, 2009). This explains why GLUD2 only correlates with cancer in brain tissue.

Figure 6: MERAV box plots comparing cancerous cell lines and normal tissue gene expression of members of the SLC glutamine transporter family. Normal tissue appears in red and cancerous cell lines in blue.

As glutamine cannot penetrate cell membranes, for glutaminolysis to occur there is a need for cancer cells to have glutamine transporters. This is why we searched for the SLC38 glutamine transporter family, as this family is considered to include some of the primary transporters of glutamine (Pochini et al.). Indeed, our queries to MERAV showed that, some members of this family, particularly SLC38A1, SLC38A2, and especially SLC38A6, are upregulated in cancerous tissues, (Figure 6). This further supports our finding that the glutaminolysis and resulting ammonia confer the selectivity of doxorubicin release from Doxil liposomes.

This is particularly useful because of the advances in metabolomics and personalized medicine that allow for a more detailed understanding of a specific patient's cancer. Miyagi et al showed in 2011 that by profiling amino acids in the blood, conclusions can be drawn about the type of cancer in the patient (Miyagi et al.). These sorts of tests can give us insight into whether the patient is the right candidate for an ammonia-triggered-release-mechanism drug by giving information on the prevalence of glutaminolysis and other relevant pathways.

Conclusions:

From the data presented in this paper, we conclude that level of glutaminolysis does correlate highly with doxorubicin release from Doxil, this correlation caused by the high concentration of ammonia that accompanies glutaminolysis, and that further, of the various metabolic pathways used by cancer cells, it is specifically the glutaminolysis that affects the doxorubicin release. Using the web-based microarray analysis tool MERAV (Shaul et al 2016) it was found that in several organs there is virtually no expression of the genes GLS and GLS2 in healthy tissue, while there is a significant expression in cancerous cell lines. This result is also supported by Spinelli et al. The high level of glutamine transporters in cancer cells (van Geldermalsen, M., et al. 2016) also supports the active glutaminolysis of cancer cells. New findings related to ammonia recycling support enhance this effect because ammonia is not cleared from the tumor as a waste product. With new tests able to detect levels of glutaminolysis in tissue, this discovery could greatly help the use of precision medicine to determine which patients can most benefit from Doxil and similar nano-liposomal formulations based on APIs which are amphipathic, weak bases that are remotely loaded by trans-membrane ion and/or pH gradients such as vinca alkaloids and camptothecins.

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Conflict of Interests

The authors declare no conflicts of interest. For a signed statement, please contact the journal office: editor@precisionnanomedicine.com

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Supporting Information

Figure S1: MERAV graphs of gene expression of GLS by organ. Red represents normal tissue and blue represents cancer cell lines. From left to right and top to bottom, organs represented are brain, breast, liver, lung, and kidney, for each gene.

Figure S2: MERAV graphs of gene expression of GLS2 by organ. Red represents normal tissue and blue represents cancer cell lines. From left to right and top to bottom, organs represented are brain, breast, liver, lung, and kidney, for each gene.

Figure S3: MERAV graphs of gene expression of GLUD1 by organ. Red represents normal tissue and blue represents cancer cell lines. From left to right and top to bottom, organs represented are brain, breast, liver, lung, and kidney, for each gene.

Figure S4: MERAV graphs of gene expression of GLUD2 by organ. Red represents normal tissue and blue represents cancer cell lines. From left to right and top to bottom, organs represented are brain, breast, liver, lung, and kidney, for each gene.