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Pre-Clinical and Clinical Data Confirm the Anticancer Effect of Deuterium Depletion

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ABSTRACT

The two stable isotopes of hydrogen, protium (^{1}H) and deuterium (^{2}H) differ in their physicochemical nature. Deuterium-depleted water (DDW) significantly inhibited the growth rate of different tumor cell lines in culture media and xenotransplanted MDA-MB-231, MCF-7 human breast adenocarcinomas and PC-3 human prostate tumors *in vivo*. The apoptosis-triggering effect of DDW was demonstrated both *in vitro* and *in vivo*. The anti-cancer effect of D-depletion was also confirmed in a double-blind, randomized, 4-month-long, human phase II clinical trial on prostate cancer. D-depletion, as an adjuvant, caused 3-7 fold increases of median survival time (MST) in lung cancer, two-fold in advanced breast cancer and it also effectively prevented recurrences of early stage breast cancer. It is suggested that the cell cycle regulating system is able to recognize the changes in the ^{2}H/^{1}H ratio. Two key events takes place in the cell at the same time: the binding of growth hormone to the receptor activates the H^+-transport system, which preferentially eliminates H^+, resulting in an increased ^{2}H/^{1}H ratio, which is essential to start cell division; the properly working mitochondria, the terminal complex of mitochondrial electron transport chain reducing molecular oxygen to DDW, which reduces the ^{2}H/^{1}H ratio and inhibits the cell growth. The balance between the activated H^+-transport system and the DDW producing mitochondria which determine the ^{2}H/^{1}H ratio in the cells is proposed as the key mechanism to regulate cell growth.

Keywords: Deuterium depleted water, DDW, Cell growth, Clinical, Cancer

INTRODUCTION

Cancer is one of the leading causes of death worldwide. It is expected that annual cancer cases will rise from 14 million (2012) to 22 million within the next 2 decades which necessitates new targets, new working hypotheses in order to develop affordable effective cancer treatments to control cancer epidemics. Although Warburg proposed in the early '30 of the last century that irreversible damage to respiration was the prime cause of cancer [1-3], after the discovery of oncogenes in 1971, cancer research shifted to regard cancer as a genetic disease. The cancer as a metabolic disease linked to oncogenesis with pentose cycle metabolism and gene clustering in 1998 using modern targeted ^{13}C-glucose fate association studies was re-disovered [4] for drug development [5].

The submolecular non-genomic theory of cancer dates back by half a century and was proposed by the 1937 Nobel laureate Albert Szent-Györgyi in Medicine. His theory links to abnormal charge transfer and permittivity, as well as limited electron carrying by methyl glyoxal, proteins and ascorbic acid with cancer [6]. Submolecular mechanisms offer very precise and relatively simple reaction

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architectures to regulate cell growth, where hydrogen and deuterium (^{2}H) showed prominent growth regulatory effects in a study performed by Somlyai *et al.* [7]. The work of Somlyai readily offered explanations for the increase in tumorigenicity of human fibroblasts expressing an ATPase dependent yeast proton ($^1H^+$) pump with strong deuterium discrimination properties [8], suggesting that the changing deuterium/hydrogen (^{2}H/^{1}H) ratio has a key role in regulating cell growth and other biochemical processes.

The studies carried out with deuterium depleted water (DDW), modifying the ^{2}H/^{1}H ratio, is considered as a new concept to develop drugs to treat cancer.

DEUTERIUM

Deuterium (^{2}H) was discovered (1931) by Harold C. Urey for which he was awarded the Nobel Prize for Chemistry in 1934. It has been known for decades that, due to the mass difference between ^{1}H and ^{2}H, molecules with a ^{2}H-content behave differently in chemical reactions [9,10]. For example, if a chemical bond contains deuterium instead of hydrogen, during the chemical reaction this bond splits approximately 6 to 10 times more slowly. If hydrogen is replaced by deuterium not in the splitting bond but at a more distant point of the molecule, chemical reactions also slow down to a significant degree. Nucleo-magnetic resonance experiments clearly reinforce that the presence of deuterium also has an effect on distant points of a given molecule, greatly influencing the behavior in chemical reactions of the molecule [11].

DEUTERIUM IN A LIVING ORGANISM

The chemical difference between deuterium and hydrogen is manifested in biological systems as well. For several decades heavy water has been used in great concentrations during experiments and it was observed that this significantly influenced processes occurring in the given biological system [9,10,12,13]. In these experiments it was stated that *e.g.* the growth of tobacco plants slowed dramatically due to increased ^2H_2O concentration. A dramatic effect was observed in the case of the mold fungus *Aspergillus niger*. This, as indicated by its name, is a black-colored fungus, which, in a heavy water medium turns alabaster white which means that the fungus is unable to produce the pigment responsible for the black color. Research carried out with mice and rats showed that in mammals, as opposed to simpler living beings, hydrogen cannot completely be substituted by deuterium: animals endure an approximately 25 ^{2}H-percent of body fluid, which can be reached by drinking heavy water of 30 percent deuterium content.

All these support the generally accepted view that the structure of heavy water is “stronger” than that of normal water. Part of the hydrogen present in living organisms H atoms bound to oxygen, sulfur, and nitrogen in a heavy water medium is quickly substituted by deuterium. In this way, hydrogen bonds responsible for the stability of proteins become deuterium bonds, but deuterium bonds are stronger than hydrogen bonds; this, in turn explains why proteins in heavy water are more stable when exposed to denaturalization and conformational changes [10].

The ^{2}H-content of living organisms on earth is basically defined by the ^{2}H-content of ocean waters and the evaporating precipitation in the form of rain and snow. In middle Europe the ^{2}H-content of surface waters is about 145 ppm (with a minimal fluctuation), *i.e.* out of a million hydrogen atoms, 145 deuterium atoms can be found as opposed to values of 155 ppm in the equatorial zone or 135-140 ppm in the northern part of Canada.

Measuring the range of ^{2}H-content at several hundred points of the globe, it can generally be stated that the ^{2}H-content of precipitation decreases when nearing the North and South poles and from the oceans towards the continental inland, as well as in proportion to height above sea level [14].

This observation can be explained by the difference in steam pressure between H_2O and ^2H_2O (or $^1H^2HO$) [15]. If the 150 ppm value of ^{2}H-concentration is expressed in terms of mM concentration, the concentration of ^2H_2O in natural water is 8.4 mM. This as deuterium is present in natural water mainly in the form of $^1H^2HO$ in fact means a 16.8 mM $^1H^2HO$ concentration.

Taking into consideration that 60 percent of an adult’s body is made up of water, and also considering that organic compounds other than water also contain deuterium, we determine that the ^{2}H-concentration of our body is somewhere between 12 and 14 mM. In contrast, human

blood contains approximately 2.24-2.74 mM calcium, 0.75-1.2 mM magnesium and 5.0-5.1 mM potassium. Considering the facts mentioned above, the question as to what role deuterium plays in biological systems becomes obvious: the presence of certain elements (calcium, magnesium, potassium) which occur in much lower concentrations in human blood and within a narrow range of concentration are indispensable for biological functioning.

LIVING ORGANISM CAN DIFFERENTIATE BETWEEN D AND H

Beyond physical fractionating, it has also been known for more than three decades that in different biological systems and, within them, in certain molecules, the $^2H/^1H$ ratio may significantly change in contrast to the $^2H/^1H$ ratio of surrounding waters. Depending on *e.g.*, whether the plant fixes carbon dioxide from the air *via* C3 or C4 pathways, the extent of the decrease in ^2H-concentration may differ or, in plants belonging to the so-called CAM group, in certain circumstances ^2H-enrichment (concentration) occurs [16]. This means that by defining the ^2H-concentration of a plant, we can tell which of the above groups it belongs to regarding photosynthesis. The refined and sensitive character of biological processes is also shown by the fact that in algae, during processes occurring by light, the cell differentiates between the two isotopes of hydrogen, whereas no such discrimination is made in dark [17]. From the point of view of our work, the recognition that the ATP-ase enzyme of yeast also discriminates between the two isotopes of hydrogen [8], is of major importance. The discrimination is manifested in the fact that the enzyme does not accept deuterium as a substrate, only hydrogen [8]. If with other living beings it is also proved that such a selectivity exists. This means that during energy-gaining processes there is a possibility for the $^2H/^1H$ ratio to significantly change in the cell or in certain organelles of the cell, such as mitochondria.

It is obvious that the biological effect of deuterium has been studied intensively until the early 90's always in high concentrations, disregarding the concentration of naturally occurring deuterium, did not assign any significance to naturally occurring deuterium.

D-DEPLETED CULTURE MEDIUM INHIBITED THE GROWTH OF CANCER CELL LINES AND TUMOR GROWTH IN XENOTRANSPLANTED MICE

In order to investigate the possible role of naturally occurring ^2H in cell cycle regulation, numerous studies have been conducted on cell lines using media prepared with deuterium-depleted water (DDW).

The first investigations in the regulation of cell division were carried out *in vitro* with different cell lines (L_{929}; MCF-7; A4; 416B) more than 20 years ago [7,18]. It was found that in a medium of lower than natural ^2H-content, cell division started after a delay of 5-10 h, however, only had a minimal effect on subsequent growth. Results revealed that cells recognize the lack of deuterium but quickly adapt themselves to the new medium. This also shows that the stoppage or slowing of cell division is caused by the difference in ^2H-concentration. When the cells were exposed to a DDW medium for several hours, the difference between the control and the DDW culture medium decreased significantly. Our results were later reinforced by investigations carried out in the laboratories of Oncotech Incorporation, in Irvine, California. In the first series of experiments, scientists followed the incorporation of H^3-thymidin into DNA after the change of medium in PC-3 (prostate), MCF-7 (breast) and M14 (melanoma) cell lines. Following deuterium depletion, inhibition was experienced in all cell lines. It is important to note that the sensitivity of cell lines was different, manifesting itself in the fact that, in the case of melanoma cell lines, inhibition was maintained for 6 hours only, while in prostate cell lines it lasted for 24 hours. In the case of breast cell lines, inhibition lasted for 48 hours. In all cases, inhibition was stronger when cells synchronized in G_0/G_1 phase were exposed to DDW culture medium. The rate of inhibition was around 20 percent [18].

In the above cases cells were exposed to a decreased ^2H-concentration on one occasion only. For modeling the process in the human body, which means a decrease in ^2H-concentration lasting for several months and occurring daily, we later had experiments carried out in the course of which the D-concentration of the culture medium was decreased not in a single step but in 2-5 steps (150-60-55-51-46-42 ppm) within 24-72 h. These experiments were

carried out *in vitro* with primary human breast and ovary tumor cells. Results showed that the more the ^{2}H-concentration was decreased, the greater the inhibitory effect, reaching, at the end of the third day, a value of 40 percent. This modeled experiment clearly demonstrates that with the continuous decrease in ^{2}H-level, the inhibition of cell division can not only be maintained for a longer time but can also be increased. The inhibitory effect of low D-concentration of media *in vitro* also was confirmed by other scientists [19].

We wish to stress that in a normal myometrial cell line, examined simultaneously, the DDW medium only slightly influenced the proliferation rate of cells. To explore the sensitivity of cell for ^{2}H-depletion, recently the most advanced technology, the label-free, real time detection technology, using the xCELLigence RTCA system (Roche Applied Sciences) was used to confirm the earlier published results. In order to investigate the possible correlation between the ^{2}H-concentration and the inhibitory effect, media containing ^{2}H at different concentrations (150 ppm, 135 ppm, 125 ppm, 115 ppm, 105 ppm, 85 ppm, 65 ppm and 40 ppm) were applied in cancer cell lines of A549, MCF7 and HT199. In each of the cell line, a clear correlation was found; the lower the ^{2}H-concentration, the lower the growth rate [20].

In order to investigate the effect of the gradual decrease ^{2}H-concentration, which occurs in human body when normal water intake is replaced with DDW, the gradual ^{2}H-depletion in the culture media was applied in every 8 hours. In the first set of measurements the decrease introduced repeatedly during culturing the cancer cells was 0 ppm (control), 5 ppm, 7.5 ppm or 10 ppm, and it resulted in a significant decrease of cell growth compared to control, but the 5 ppm caused the same inhibitory effect as 10 ppm (xCELLigence RTCA system is a microelectronic cell sensor method that features microelectrodes integrated in the bottom of a microtiter plate (96-well E-plate) and measures continuously the changes in the adhesion and proliferation of the observed cell lines). The presence of the cells on top of the electrodes affected the local ionic environment, leading to an increase in the electrode impedance. The more cells are attached to the electrodes, the larger is the increase in electrode impedance. The dimensionless parameter called Cell Index (CI) is able to describe the viability of the cell and the cell count.

In order to determine the lowest degree of decrease in ^{2}H concentration which is able to trigger inhibitory effect, in the next study the ^{2}H-concentration of the media was decreased by 0 ppm (control), 1 ppm, 2.5 ppm or 5 ppm in every 8 h. The CI of A549 lung adenocarcinoma cells was lower in comparison to the control treatment after the second ^{2}H-depletion by 1 ppm, 2.5 ppm or 5 ppm, and after the 3rd decrease the dose dependency was evident and could be detected during the whole monitoring period until 90 hours. The gradual ^{2}H decrease only resulted in a slight inhibition in MCF7 cells which suggested the ability for adaptation to the modest decrease in ^{2}H concentration, while the HT199 melanoma cells were also sensible to the method, but a significant inhibition of the HT199 cells was measured only after the 4th treatment (32 h) [20].

The consumption of DDW as drinking water resulted in complete or partial tumor regression in mice xenotransplanted with MDA-MB-231 or MCF-7 human breast adenocarcinomas or PC-3 human prostate tumors [7, 21]. The apoptosis-triggering effect of DDW was detected both *in vitro* and *in vivo* [18]. ^{2}H-depletion also exerts an influence on protooncogenes and tumor suppressor genes, and the expression of c-myc, Ha-ras and p53 genes induced by carcinogen exposure was significantly weakened when the animals were given DDW to drink [22]. The DDW also was tested on dogs and cats having different types of cancer as part of drug registration to get approval as an anti-cancer drug for veterinary use [18]. The drug registration was closed in 1999 and the DDW with 25 ppm ^{2}H-concentration was approved by the Hungarian authority as a drug to treat household pets. In the last 17 years several pets were treated, the response rate was over 70%, the complete remission reaches 50% of the treated animals.

PROSPECTIVE (PHASE 2) AND RETROSPECTIVE CLINICAL STUDIES PROSTATE CANCER

So far only one phase 2, prospective, double blind, randomized human study has been reported [23]. The four-month-long phase 2 clinical trial was conducted (under the permission of the Hungarian Institute of Pharmacology: No. 5621/40/95) in four Hungarian trial sites. The daily water intake of prostate cancer (PC) patients was replaced with

DDW of 85 ppm ^2H concentration (treated group), or remained normal with 150 ppm ^2H (placebo group). The protocol contained no restrictive requirements with respect to the conventional treatments. Forty-four patients were evaluated in the Intention-to-Treat analysis (ITT Population), 22 patients were involved in the treated and 22 patients in the placebo group. Thirty-three patients completed the trial in full accordance with the protocol requirements (PP Population). Seven patients in the treated group, and one patient in the placebo group achieved partial response (PR) ($p < 0.05$). No change (NC) was verified in 11 patients in the treated, and in 13 patients in the placebo group (non-significant difference). Progression of disease (PD) was diagnosed in 4 cases in the treated group and in 8 patients of the control group. Because of the duration of the clinical trial (only 4 months), complete response (CR) was not verified in any of the patients during the study. Prostate volume decreased in 18 out of 22 patients in the treated arm. No change was detected in one patient while the prostate volume increased in two subjects. There was no available data on the prostate size in one patient. In the placebo group, prostate size decreased in 11 patients, no change was recorded in 5 patients, and increased prostate size was found in another 5. The net decrease was 160.3 cm^3 in the treated, and 54 cm^3 in the control group ($p = 0.0019$). It became evident that the PSA and prostate size were changing concomitantly in numerous cases. Cumulative PSA showed decrease in the test groups in the four-month-long study. The baseline value was 406.4 ng ml^{-1} and 521 ng ml^{-1} in the treated and placebo group, respec-tively. By the end of the trial the cumulative PSA value decreased to 80.3 ng ml^{-1} in the treated and 277.4 ng ml^{-1} in the placebo group. Survival data were obtained during the three-year-long extended follow-up: in the first year 2 patients (9.1%) died in the treated group and 9 patients (40.9%) in the placebo group ($p = 0.034$, Fisher's exact test). After a two-year follow-up, 7 patients died in the treated, and 12 in the placebo group, by the end of the third year the ratio was 8:12. All these data supported the findings of preclinical studies which indicated the anticancer effect of deuterium depletion.

Since 1994 deuterium depleted drinking water was available to purchase in Hungary, allowed to follow a large group DDW consuming cancer patients retrospectively. To get additional evidence for the anticancer effect of ^2H-depletion, homogenous cancer population was constituted in order to calculate median survival time (MST) and compare to historical control. The database contains 91 prostate cancer patients [23]. Forty-five patients having no metastasis, 46 patients had distant metastases at the time they started to consume DDW. According to the Kaplan Meier curve of 45 patients without distant metastasis, due to the extremely long survival, the data were not suitable for calculating MST (only 4 patients (9%) died). Out of the 46 patients having distant metastases, the subgroup of 20 patients whose bone metastases were verified within one year from the diagnosis of PC was separated. On this subgroup, we found the MST 65.2 months, which shows a three-fold increase to historical control (15-20 months) [23].

LUNG CANCER

The first retrospective study with lung cancer patients having brain metastasis was published in 2008 [24]. A series of 4 case histories was retrospectively evaluated. The patients were diagnosed with brain metastasis derived from a primary lung tumor and started consuming DDW at the time of or after the diagnosis of the brain metastasis, which was either inoperable or the surgical intervention did not result in complete regression. The primary objective was survival. Patients were consuming DDW for at least 3 months. The deuterium level was contiously lowered by 10 to 15 to 20 ppm in every 1 to 2 months and thus a gradual decrease was maintained in the ^2H-concentration in the patient's body. DDW was integrated into conventional treatments as an adjuvant resulted in a survival time of 26.6, 54.6, 21.9 and 33.4 months in the 4 patients, respectively. The brain metastasis of 2 patients showed complete response (CR), whereas partial response (PR) was detected in 1 patient, and the tumor growth was halted (no change or NC) in 1 case. One of the patients with complete response had 11 years survival, lived for several years after the paper was published.

The anticancer effect of ^2H-depletion on lung cancer patients also was confirmed evaluating the data from 129 lung cancer patients [25]. The study revealed that the MST of 27 lung cancer patients with brain metastasis was 27.1 months, which also represents a several fold increase in survival comparing to historical control.

In the entire population of lung cancer patients in Hungary between 2002 and 2005, the MST of males was 7.5 months, with a 10% 5-yr survival probability, and in females the MST was 11.3 months, with a 5-yr survival probability of 20.5% [25]. In the study, which lasted from 1993 to 2010, the MST was 25.8 months in males, 74.1 months in females, and 33.7 months in both sexes overall. The 5-yr survival probabilities were 19%, 52%, and 33% in males, females, and both sexes, respectively.

BREAST CANCER

To get further insight into the effect of ^{2}H-depletion in clinical application the data of 232 breast cancer patients were carefully evaluated [26]. A formula was introduced to calculate deuterium depletion unit (DdU) in order to compare the different dose of treatment depending on the body weight of patients, the daily volume of DDW and the ^{2}H-concentration of the DDW:

$$Dose\,(DdU)\,\frac{150\,(ppm) - D\ concentration\ of\ \ DDw\,(ppm)}{body\ weight\ (kg)} \times volume\ of\ \ DDW\left(\frac{L}{day}\right)$$

The DdU was in the range of 0.02 to 4.71 in the population during the follow-up, and the response to DDW treatment was determined at 85 time points in the 74 patients being in stage IV. The data showed in 60% of the patients where the DdU was higher than "1" CR or PR was confirmed, if the DdU was lower than "1" NC or PD was diagnosed. The MST was 52 months (4.3 years) for 74 patients with advanced cancer, which shows a 2-fold increase compared to historical control.

One of the most striking observation of the paper was related to the subgroup of breast cancer population (48 patients) which started to consume DDW after the operation in remission; only one out of these 48 patients died during the cumulative follow-up period of 221.1 years (median follow-up time: 32 months).

Prevention of relapses and/or development of distant metastases is a crucial aspect of breast cancer, even decades after the first diagnosis and successful treatment. Further subgroups were formed according to the frequency of DDW treatment. A single DDW treatment was administered to 179 patients whereas 53 patients repeated the cure at least once. Patients who were treated with DDW at least twice had an MST of 293 months (24.4 years) whereas those who received a single DDW treatment had an MST of 108 months (9 years) [26].

SUPPOSED MECHANISMS OF ACTION

It was suggested that the cell cycle regulating system is able to recognize the changes in the ^{2}H/^{1}H ratio [7]. Two key events take place in the cell at the same time: the binding of growth hormone to the receptor activates the H^+-transport system, which preferentially eliminates H^+, resulting in an increased ^{2}H/^{1}H ratio within the cells which can lead to cell multiplication [7,27], meantime the properly working mitochondria, the terminal complex of mitochondrial electron transport chain reducing molecular oxygen to DDW, which reduces the ^{2}H/^{1}H ratio and inhibits cell growth [28-30]. The mitochondria-produced DDW affects gluconeogenesis as well as fatty acid oxidation. In the former, the DDW is thought to diminish the deuteration of sugar-phosphates in the DNA backbone, helping to preserve stability of hydrogen bond networks, possibly protecting against aneuploidy and resisting strand breaks, occurring upon exposure to radiation and certain anticancer chemotherapeutics. The activated H^+-transport system and the DDW producing mitochondria which determine the ^{2}H/^{1}H ratio in the cells is proposed as the key mechanism in cancer prevention and treatment using natural ketogenic diets, low deuterium drinking water, as well as DDW production as the mitochondrial downstream mechanism of targeted anti-cancer drugs such as Avastin and Glivec. The role of ^{2}H in biology is a potential missing link to the elusive cancer puzzle.

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