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Impressive Suppression of Colon Cancer Growth by Triple Combination SN38/EF24/Melatonin: "Oncogenic" *Versus* "Onco-Suppressive" Reactive Oxygen Species

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Abstract. *Background/Aim: The study aimed to investigate the effect of multi-targeted combinations (SN38/EF24; SN38/EF24/melatonin) on the growth of colon cancer in experimental animals and their impact on the ratio "oncogenic"/"onco-suppressive" reactive oxygen species (ROS) – a crucial factor for triggering carcinogenesis, as well as for development of effective therapeutic strategies. Materials and Methods: The experiments were conducted on colon cancer-grafted mice – non-treated, SN38/EF24-treated and SN38/EF24/melatonin-treated within 22 days. The balance between different types of ROS was measured in vivo by nitroxide-enhanced magnetic resonance imaging (MRI), as well as on isolated tissue specimens by conventional analytical tests. Results: Both combinations significantly suppressed the tumor growth. Impressive anticancer effect was observed in SN38/EF24/melatonin-treated mice – almost complete destruction of the tumor. Both types of ROS (superoxide and hydroperoxides) were elevated in cancer, but the MRI data suggest that the ratio between them tends towards superoxide. SN38/EF24 decreased the level of superoxide, but did not affect the level of hydroperoxides in the cancerous tissue, while SN38/EF24/melatonin decreased the level of superoxide below the control and increased significantly the level of hydroperoxides. Conclusion: The most important observations are that: (i) colon cancer was characterized by a vicious cycle, that ensures a permanent domination of "oncogenic" ROS (as superoxide) over "onco-suppressive" ROS (as hydrogen peroxide); (ii) the anticancer effect of the triple combination EF24/SN38/melatonin was accompanied by decreasing "oncogenic" and increasing "onco-suppressive" ROS; (iii) the ratio between both types of ROS could be a new onco-target for combined therapy; and (iv) nitroxide-enhanced MRI is a valuable tool for analyzing of this ratio.*

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Colorectal cancer is the third most common cancer in the world and in the top 5 of the most life-threatening cancers. Every year, nearly 1.5 million new cases are diagnosed as having colorectal cancer, and about 25-30% of the patients die from the disease (1-2). The highest incidence rates are estimated in developed countries (*e.g.*, Australia, Western Europe, US, Japan, South Korea, Singapore), and the lowest – in south Asian countries (1-3).

Dietary constituents are the most crucial factors for development of colorectal cancer, as well as for its prevention. The low incidence of colorectal cancer in South-Central Asia is often attributed to dietary intake of curcumin, the yellow pigment in the spice turmeric of curry powder, which is widely used in almost all dishes in this region (4).

The role of curcumin as an anticancer agent has attracted attention since 2001, when turmeric extract was applied in 15 patients with colon cancer, who did not respond to standard chemotherapy agents and radiation therapy, but 5 of them responded favorably to turmeric (5). Five years later, a small but informative clinical trial by Johns Hopkins investigators showed that the combination of curcumin and quercetin reduced the number and size of ileal and rectal adenomas in patients with an inherited form of colon cancer

(6). It is important to remember that the benefits of the diet are seldom produced by a single ingredient in that diet. In this context, the combination of several ingredients could be more effective, especially when they are targeting different onco-targets.

Structural curcumin analogs have been designed to optimize the therapeutic effects of curcumin by enhancing potency, reducing side-effects, and increasing bioavailability (7-10). Diphenyl-difluoroketone (EF24) is one of the most attractive analogs among them. It shows ~8-fold greater anticancer potential than curcumin, especially against colorectal cancers and received much attention from pharmacologists in the world due to its multiple anticancer effects (11, 12). It is one of curcumin analogs most close to the anti-cancer clinical trial. A number of molecular targets in various types of cells, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), tumor suppressor protein p53, hypoxia-inducible factor 1-alpha (HIF-1α), protein kinase B (AKT), mitogen-activated protein kinases (MAPK), phosphatase and tensin homolog (PTEN) *etc*., are affected by EF24 (12-14). It was also reported that EF24 induces cell cycle arrest and apoptosis by a redox-dependent mechanism in various cancer cells: (i) by enhancement of reactive oxygen species (ROS) (in particular hydrogen peroxide) and/or decrease of glutathione (15); and (ii) by up-regulation of the cellular antioxidant response system (12). A recent study demonstrated that EF24 increases production of hydrogen peroxide and other hydroperoxides [analyzed by two dichloro-dihydro-fluorescein (DCF)-based tests], but decreases production of superoxide [analyzed by dihydroethidium (DHE)-test] in gastric cancer cells (13). DHE is considered highly specific for superoxide, while DCF derivatives are considered highly specific for hydroperoxides and mainly for hydrogen peroxide (16).

Currently, irinotecan is one of the most promising conventional chemotherapeutics against colon cancer. It is clinically applied after laparoscopic or open surgical resection in the daily medical practice. This anticancer drug is on the World Health Organization List of Essential Medicines (17). Irinotecan is a pro-drug and it is metabolized in the liver and converted to 7-ethyl-10-hydroxycamptothecin (SN38) – an active metabolite, that is a type 1 topoisomerase inhibitor. Several studies have reported that SN38 induces apoptosis in colon and gastric cancer cell lines *via* a ROS-mediated mechanism (18-20). It is important to note that all these studies are based on DCF-tests for evaluation of ROS level.

We consider most interesting and informative, studies which investigate simultaneously the effect of anticancer drugs on the level of different types of ROS – *e.g*., superoxide and hydrogen peroxide (or hydroperoxides). In this article, we would like to emphasize the importance of the ratio superoxide/hydrogen peroxide for triggering carcinogenesis, as well as for development of effective therapeutic strategies. Many data indicate that superoxide is an "oncogenic" ROS, while hydrogen peroxide is an "onco-suppressive" ROS (21-25).

Our study is also based on the concept of multi-targeted and redox-mediated anti-cancer therapy. It was designed to investigate the effect of two combinations (SN38/EF24 and SN38/EF24/melatonin) on the growth of colon cancer in experimental animals and their impact on the redox-status of cancerous and non-cancerous tissues, and particularly on the ratio "oncogenic"/"onco-suppressive" ROS. Melatonin has been selected as one of the most potent endogenous redox-modulators in mammals, exhibiting multiple signaling effects in colon cancer and synergistic cytotoxicity in combination with conventional chemotherapeutics, including irinotecan (26-32).

Tissue redox-status was measured *in vivo* on anesthetized animals, using nitroxide-enhanced magnetic resonance imaging (MRI). The method is based on the redox-cycle of cell-penetrating nitroxide derivatives and dynamics of their T1 weighted MRI contrast, which make them useful molecular sensors for visualization of tissue redox-status *in vivo*, as well as for detection of mitochondrial dysfunction and metabolic activity accompanied by overproduction of superoxide – inherent events of cancer cells and tissues (33-36). This methodology also allows distinguishing cancerous from normal tissues based on differences in their redox-status.

Materials and Methods

Chemicals. EF24, SN38, and melatonin were purchased from Sigma-Aldrich, Steinheim, Germany. Mito-TEMPO was purchased from Enzo Life Sciences, Exeter, UK. All chemicals were analytical or HPLC grade.

Animals. The experiments were conducted in accordance with the guidelines of the Physiological Society of Japan and were approved by the Animal Care and Use Committee of the National Institute of Radiological Sciences, Chiba, Japan.

The mice (male nude *Balb6*) were separated into two major groups: healthy mice (controls, n=3) and colon cancer-grafted mice (n=9). In both groups, the mice were same age, almost same weight (23±2 g), and maintained under same conditions. Colon cancer-grafted mice were separated into three sub-groups: (i) non-treated; (ii) SN38/EF24-treated; (iii) SN38/EF24/melatonin-treated, as described below.

Colon26 cells (0.5×10^5 cells in 10 μl) were inoculated subcutaneously in one hind-paw of the mouse to trigger a development of colon cancer. The mice were subjected to treatment with anticancer drugs (EF24, SN38) and melatonin on the 7th day of the inoculation, when the tumor reached a size ~75-100 mm^3.

Treatment protocol. Two combined stock-solutions were prepared: (i) SN38 (5 mg/ml) and EF24 (200 μg/ml), dissolved in dimethylsulfoxide (DMSO); and (ii) SN38 (5 mg/ml), EF24 (200 μg/ml), and melatonin (5 mg/ml), dissolved in DMSO. The stock-solutions were divided into

Eppendorf tubes (in aliquots of 500 μl) and stored at –20˚C. Everyday aliquots were thawed and used for injection.

Fifty μl of the respective stock-solution were injected subcutaneously (*s.c.*) under the tumor everyday within 22 days. The daily doses of the injected substances were: SN38 – 10 mg/kg body weight (b.w.); EF24 – 400 μg/kg b.w.; melatonin – 10 mg/kg b.w.

In vivo MRI measurements. In the end of treatment (on 23rd day), three mice from each group were subjected to MRI for evaluation of tissue redox-status. The MRI measurements were performed on a 7.0 Tesla horizontal magnet (Kobelco and Jastec, Kobe, Japan) interfaced to a Bruker Avance console (Bruker BioSpin, Ettlingen, Germany) and controlled with the ParaVision 4.0.1 program (Bruker BioSpin, Germany).

The mice were anesthetized by isoflurane (1.2%) and placed in a head or body holder (RAPID Biomedical, Germany). A respiration sensor (SA Instruments, Inc., NY, USA) was placed on the back of the mice. A temperature probe (FOT-M and FTI-10, FISO Technologies Inc., Quebec, Canada) was used to monitor the rectal temperature. The tail vein was cannulated using a polyethylene tube (PE-10, Becton-Dickinson, NJ, USA) for injection of contrast substance. The mouse was placed in the 1H- volume radio-frequency (RF) resonator (Bruker BioSpin) with surface RF receiver (RAPID Biomedical, Rimpar, Germany), which was pre-warmed using a body temperature controller (RAPID Biomedical, Rimpar, Germany). The resonator units, including the mouse, were placed in the magnet bore. The body temperature was maintained at 37±1˚C during the MRI measurements.

Five control images of the mouse body were taken before injection with the following parameters: T1-weighted incoherent gradient-echo sequence (fast low-angle shot; FLASH), repetition time=75 ms; echo time=3.5 ms; flip angle=45 degrees; field of view=3.2×3.2 cm; number of averages=4; scan time=19.6 seconds; matrix=64×64; slice thickness=1.0 mm; and number of slices=4. Hundred μl of mito-TEMPO [100 mM stock solution in 10 mM phosphate-buffered saline (PBS), pH 7.4] were injected *via* the tail vein (final concentration of mito-TEMPO – 0.4 μmol/g b.w.) 1 min 40 sec after beginning the scan. T1-weighted images were acquired continuously within approximately 14 min, at the parameters described above.

The MRI data were analyzed using ImageJ software (National Institutes of Health, MD, USA). The average value of the first five control sequences (recorded before injection) was calculated and each sequence of the kinetic measurement was normalized to this average value by division using an identical algorithm as described by Zhelev *et al*. (33).

Preparation of tissue homogenates. Mice were sacrificed and cancer tissue was removed and homogenized in 10 mM PBS (pH 7.4) at 4˚C, using digital tissue homogenizer "AsOne" (Tokyo, Japan). Tissue homogenates were adjusted to equal protein concentration (10 mg/ml) using 10 mM PBS (pH 7.4). Protein concentration was analyzed by Bradford assay. All analysis on tissue homogenates were performed simultaneously.

Dihydroethidium (DHE) assay. The major advantage of DHE-test is its ability to distinguish between superoxide and hydrogen peroxide (18, 37).

Briefly, DHE was dissolved in DMSO to 65 mM stock solution, which was diluted with PBS to prepare 50 μM DHA working solution in the day of experiment. Ten μl of 50 μM DHA were added to 100 μl of tissue homogenate. The samples were incubated for 15 min at room temperature and fluorescence intensity was detected immediately at λex=518 nm and λem=605 nm, using microplate reader (TECAN Infinite® M1000, Grodig, Austria).

OxySelect™ in vitro ROS/RNS assay. The amount of ROS/RNS was analysed using OxiSelect™ *in vitro* ROS/RNS Assay Kit – Green Fluorescence (Cell Biolabs, Inc., San Diego, CA, US). The method is based on a fluorogenic probe – 2',7'-Dichlorodihydrofluorescin DiOxyQ (DCFH-DiOxyQ). In the cytosol, the non-fluorescent DCFH-DiOxyQ is deacetylated to the non-fluorescent 2',7'-Dichlorodihydrofluorescin (DCFH). DCFH reacts rapidly with ROS and reactive nitrogen species (RNS) (predominantly H_2O_2, ROO., ROOH, NO, ONOO-) with formation of a fluorescent product – 2',7'-Dichlorodihydrofluorescein (DCF) (18). The intensity of DCF fluorescence (λ_{ex}=480 nm, λem=530 nm) is proportional to the amount of ROS in the biological sample.

The amount of ROS was calculated by calibration curve, based on DCF standard solutions in PBS. The measurements were performed on microplate reader "Tecan Infinite F200 PRO" (Tecan Austria GmbH, Grodig, Austria).

Briefly, the tissue homogenates were incubated with DCFH-DiOxyQ and subjected to ROS assay, according to the manufacturer's instructions.

Statistical analysis. The data were statistically analyzed by ANOVA using Student's *t*-test. A value of $p<0.05$ was considered significant.

Results and Discussion

Figure 1 demonstrates tumor growth in colon cancer-grafted mice without treatment (red curve) and during subcutaneous treatment with both combinations: (i) SN38/EF24 (blue curve); and (ii) SN38/EF24/melatonin (green curve). The treatment significantly delayed tumor growth in both groups. The tumor volume was about 10-times lower in SN38/EF24-treated mice – 300 mm^3 *versus* 3000 mm^3 in non-treated colon-grafted mice (Figure 1A – red curve, Figure 1B – blue curve). In SN38/EF24/melatonin-treated mice, the tumor size increased slowly within 10 days, but then decreased almost to the initial size (Figure 1B – green curve). This can be seen also from the pictures in Figure 1C. In the first few days of the treatment with SN38/EF24/melatonin, we observed an induction of inflammation in the cancerous tissue, followed by a rapid decrease in tumor size.

After the end of treatment, the mice were subjected to nitroxide-enhanced MRI for assesment of tissue redox-status. Mito-TEMPO was used as a redox-sensitive contrast substance. Mito-TEMPO is a cell-penetrating nitroxide derivative with prevalent mitochondrial accumulation (36). Two regions-of-interest (ROIs) were selected: (i) located in the tumor-free hind-paw (ROI1); and (ii) located in the tumor area of cancer-grafted hind-paw (ROI2) (Figure 2). In both ROIs, the signal increased after injection of mito-TEMPO, followed by a rapid or slower decrease. The enhancement of the MRI signal in the beginning was due to

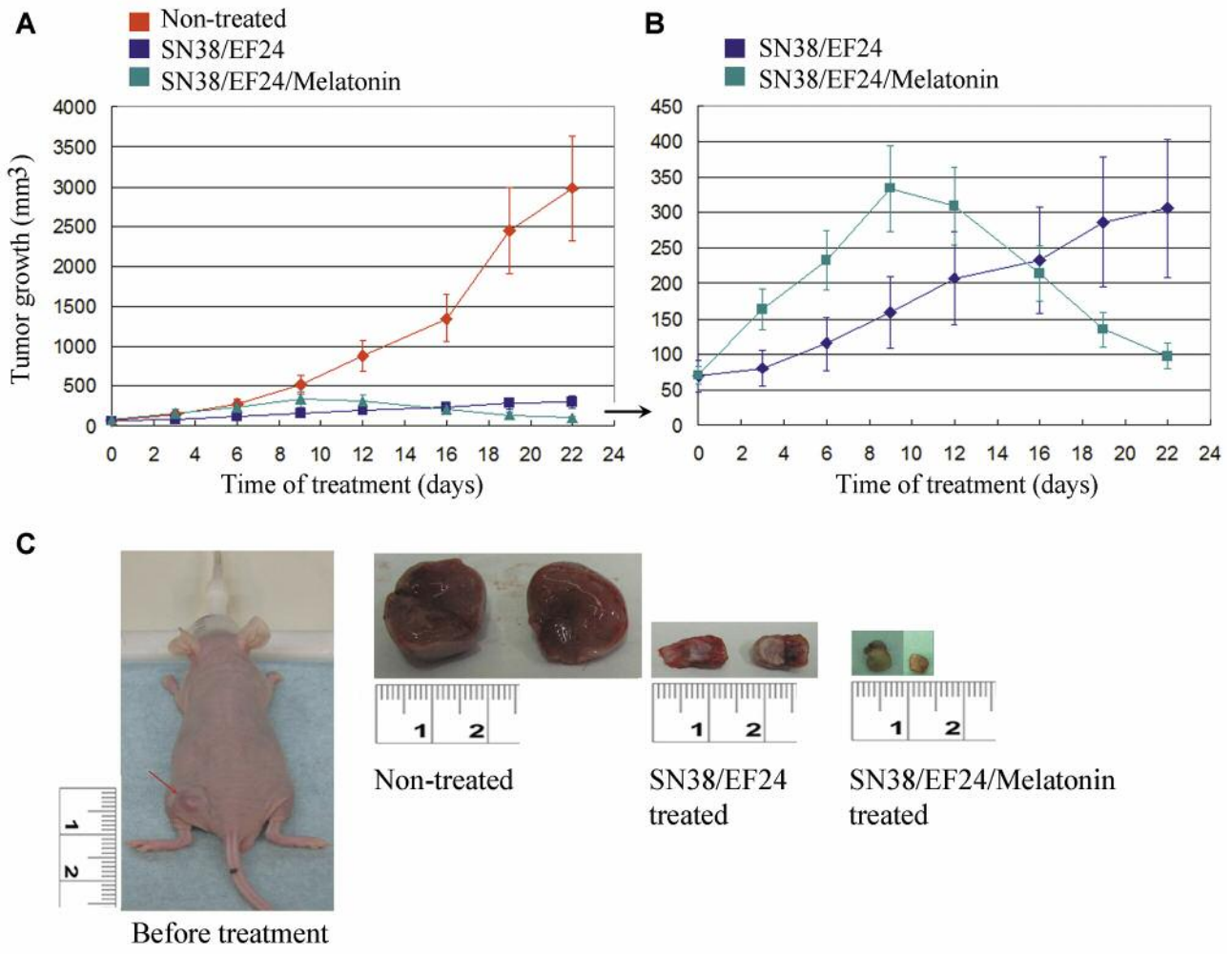

Figure 1. *Effect of SN38/EF24 and SN38/EF24/melatonin on the tumor growth in colon cancer-grafted mice. (A, B) Kinetic curves of tumor growth after treatment. The data are Mean±SD from six mice in each group. (C) Representative pictures of tumor size before treatment and in the end of treatment.*

the presence of paramagnetic nitroxide in the blood and its penetration and accumulation in the subsequent tissue, whereas the decrease was due to its reduction to diamagnetic (non-contrast) hydroxylamine, which occurs predominantly into the cells.

In ROI1 of healthy (tumor-free) mice, the half-life of nitroxide-enhanced MRI signal ($\tau_{1/2}$) is approximately 4 min and the duration of the signal is very short (~6-7 min) (Figure 2A, black curve). The profile of the histogram indicated a high reducing capacity of the healthy tissues (*e.g.*, muscle) in respect to mito-TEMPO. In ROI1 of non-treated cancer-grafted mice, the signal increased, reached a plateau, and then decreased very slowly without reaching baseline within 14 min ($\tau_{1/2}$>14 min; p<0.001 *versus* healthy mice) (Figure 2A, red curve). This histogram indicates a high oxidative capacity of the non-cancerous tissues in tumor-free hind-paw. In ROI2 (tumor area) of non-treated cancer-grafted mice, the signal increased after injection of mito-TEMPO and reaches a plateau without decrease within 14 min (Figure 2B, red curve). This histogram also indicates a high oxidative capacity of cancerous tissue.

These data are in agreement with our previous studies, demonstrating that cancerous and normal tissues could be distinguished based on their redox-status, using nitroxide-enhanced MRI (33, 34, 38). Moreover, all these studies (including current) indicate that oxidative status of non-cancerous tissues (even those distant from the primary tumor locus) increases in advanced stage of cancer (final stage, late-stage of cancer). Non-cancerous tissues become susceptible to oxidative stress and damage and should be considered a potential therapeutic target.

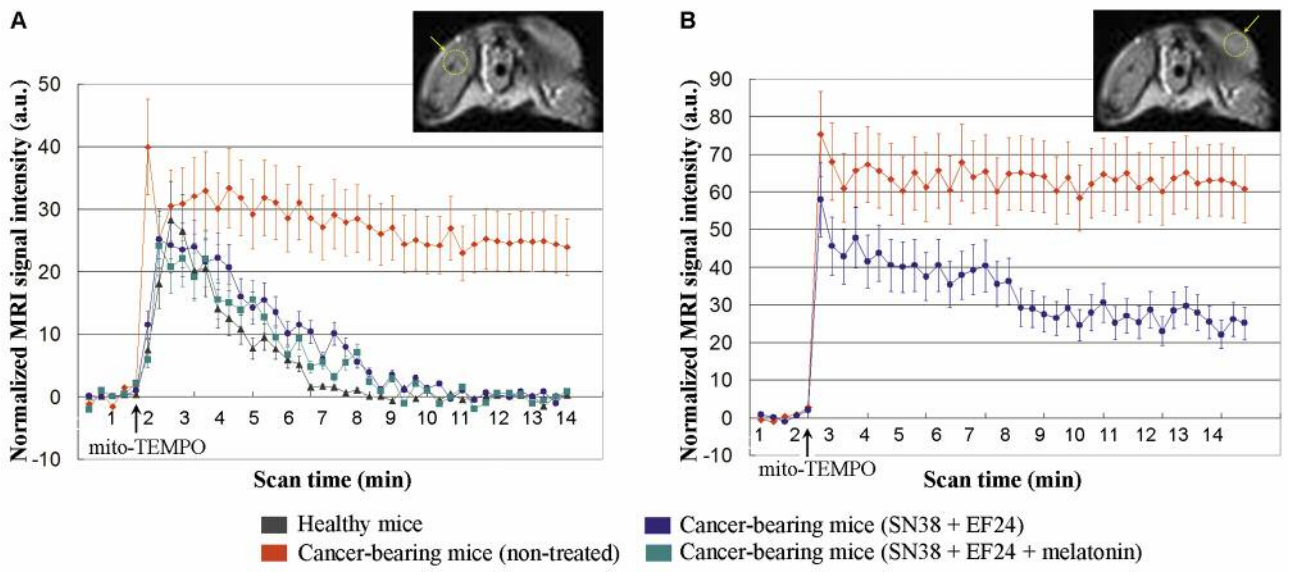

Figure 2. *Effect of SN38/EF24 and SN38/EF24/melatonin on nitroxide-enhanced MRI signal in healthy and colon cancer-grafted mice. (A) The region-of-interest is located in the tumor-free hind-paw (ROI1). (B) The region-of-interest is located in the tumor area of the tumor-bearing hind-paw (ROI2). The data are Mean±SD from three mice in each group.*

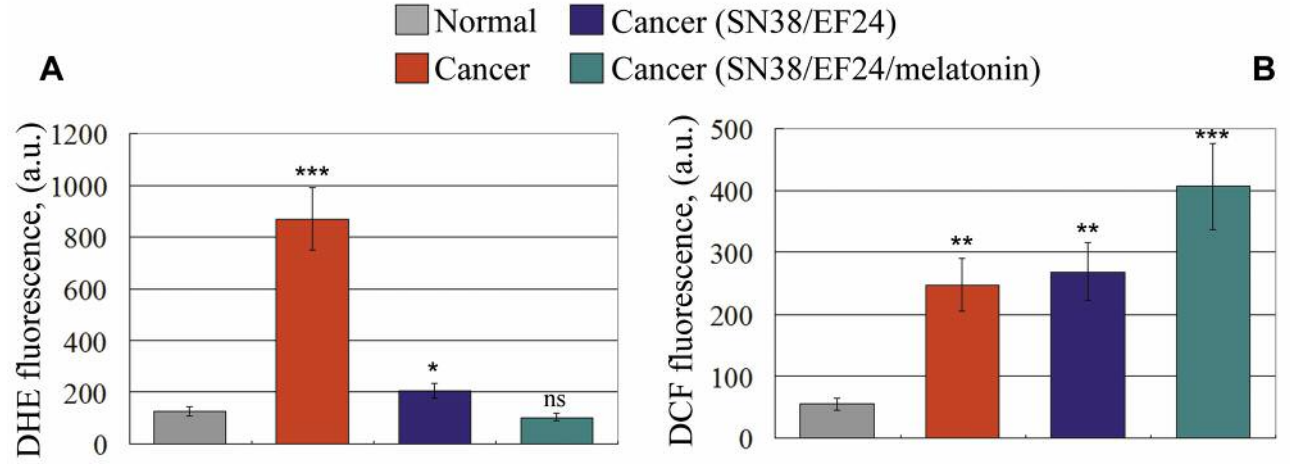

Figure 3. *Levels of ROS/RNS in normal muscle tissue of healthy mice (control) and cancerous tissue of untreated, SN38/EF24-treated and SN38/EF24/melatonin-treated colon cancer-grafted mice. (A) Superoxide level, determined by dihydroethidium (DHE test). (B) Level of hydrogen peroxide, hydroperoxides and reactive nitrogen species (RNS), determined by OxiSelect™ In Vitro ROS/RNS Assay (DCF test). The data are Mean±SD from three mice in each group. *p<0.05; **p<0.01; ***p<0.001 versus control group – non-treated healthy mice.*

What is the effect of treatment on tissue redox-status?
In ROI1 of SN38/EF24-treated and SN38/EF24/ melatonin-treated cancer-grafted mice, the histograms were similar to that in healthy mice – $\tau_{1/2}$ of nitroxide-enhanced MRI signal was slightly higher (~5-6 min) and the duration was slightly longer (~8-9 min), compared to non-treated healthy mice (Figure 1A, blue and green curves).

In ROI2 of SN38/EF24-treated mice, the histogram had a different profile – the signal increased, reached a peak, and then decreased slowly within 14 min without reaching

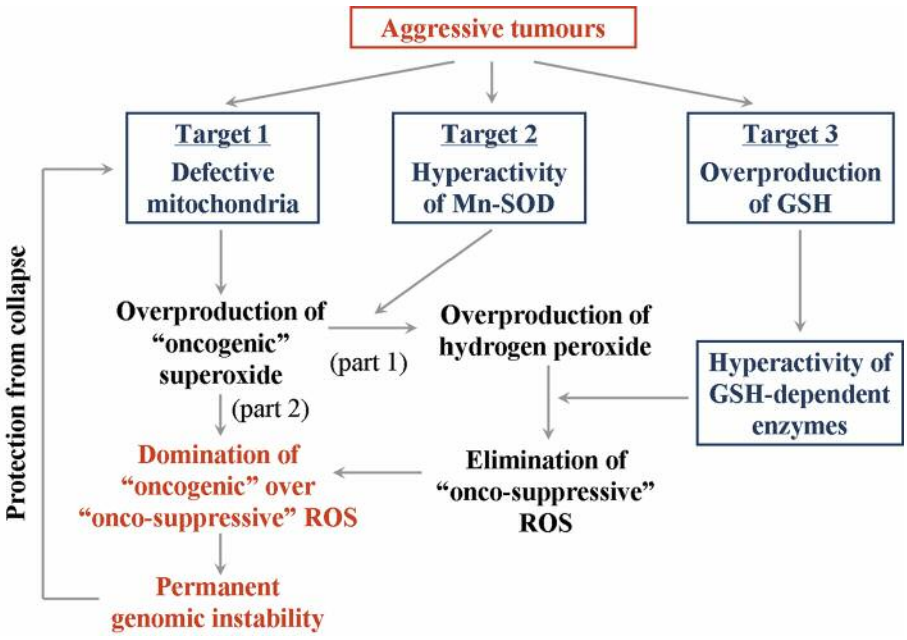

Figure 4. *"Oncogenic" versus "onco-suppressive" ROS in aggressive tumours – vicious cycle and therapeutic targets.*

baseline (Figure 2B, blue curve). The normalized intensity was lower in SN38/EF24-treated cancer-grafted mice than in non-treated mice. The histogram indicates a decrease of oxidative capacity of cancer tissue of SN38/EF24-treated mice, compared to non-treated cancer-grafted mice.

In SN38/EF24/melatonin-treated mice, it was very difficult to locate precisely the tumor (ROI2) on MRI, as well as to measure nitroxide-enhanced signal in this area due to its small size and large cicatrix as a result of inflammation-like process, observed in the tumors of this group.

Briefly, the MRI data demonstrated that: (i) the tissues of healthy mice are characterized by a high reducing capacity; (ii) the tissues of cancer-grafted mice are characterized by a high oxidative capacity; (iii) both combinations (SN38/EF24 and SN38/EF24/melatonin) decreased the tissue oxidative capacity in cancer-grafted mice.

Many studies have shown that various intra- and extracellular reducers and oxidizers can convert (directly or indirectly) paramagnetic nitroxide radical into diamagnetic forms – hydroxylamine and oxoammonium (39-41). Both diamagnetic forms are "superoxide dismutase mimetics" (41). The interaction of oxoammonium with superoxide occurs at pH<4.5, whereas in living cells and tissues, the oxoammonium is reduced to hydroxylamine by NAD(P)H (41). Recently, we established that in cell-free systems, as well as in cultured cells only interaction of hydroxylamine with superoxide, but not with hydrogen peroxide, restores nitroxide radical and its MRI contrast (35, 36). Thus, nitroxide-enhanced MRI allows visualization of the balance between superoxide and hydrogen peroxide in vitro and *in vivo*, which may be useful for elucidating the molecular nature of the cell-signaling regulation and its disturbance in a variety of pathologies, including carcinogenesis.

Therefore, our present MRI data (*in vivo*) indicated that: (i) There was overproduction of superoxide in the cancerous tissue (Figure 2; ROI2); (ii) There was also overproduction of superoxide in non-cancerous tissues of cancer-grafted mice. Obviously, these tissues are under oxidative stress and could be susceptible to damage; (iii) The combinations SN38/EF24 and SN38/EF24/melatonin decreased the level of superoxide in cancerous tissue and normalized the level of superoxide in the surrounding tissues of cancer-grafted mice.

Both types of ROS (superoxide and hydrogen peroxide) can be elevated in cancer, but the MRI data suggest that the ratio superoxide/hydrogen peroxide tends towards superoxide.

To confirm this assumption, we analyzed the levels of superoxide and hydrogen peroxide in isolated tissue specimens (from healthy and cancer-grafted mice), using conventional analytical tests – DHE and DCF-based (Figure 3). Both types of ROS were increased in cancerous tissue, compared to the normal tissue of healthy mice. SN38/EF24 decreased the level of superoxide, but did not affect the level of hydroperoxides in the cancerous tissue. SN38/EF24/melatonin decreased the level of superoxide (even below the control) and increased significantly the level of hydroperoxides in the cancerous tissue.

Aykin-Burns *et al*. have also reported that the levels of superoxide and hydrogen peroxide are substantially higher in cancer cells than in normal cells (42). The authors have established that cancer cells (derived from human colon and breast cancers) are characterized by a significantly higher oxidation of DHE (2-20 fold) and carboxy-dichlorodihydrofluorescein diacetate ($CDCFH_2$) (1.8-10 fold), compared to normal human cells (*e.g*. colon epithelial cells, colon fibroblasts, and mammary epithelial cells) (42).

Based on these data, we may speculate that the level of superoxide is higher than that of hydrogen peroxide in cancer cells and tissues. However, the conventional analytical tests can not evaluate which type of ROS dominates, because the level of superoxide can not be measured in absolute units (16). It is impossible to calculate the molar concentration of superoxide in cell/tissue specimens (using DHE test) and to compare with the data for hydrogen peroxide, obtained in absolute units (moles; using DCF tests). In this context, our MRI methodology provides greater opportunities than conventional analytical tests, since it allows to assess which type of ROS dominates, due to the opposite effects of superoxide and hydrogen peroxide on proton relaxivity.

When talking about ROS-mediated anticancer effects, it is necessary to consider and specify the type of ROS. Otherwise, misinterpretation of the data is very likely, because the levels of different types of ROS can change simultaneously in different directions – to increase, to decrease, or to be at constant level. To avoid that, it is obligatory to use a set of comparable analytical tests for the detection of different types of ROS in the biological object.

The ratio between main intracellular ROS (*e.g*. superoxide and hydrogen peroxide) could be crucial for the choice at cell signaling – proliferation or apoptosis, as well as for the metastatic potential of cancer cells. Dual effects of superoxide and hydrogen peroxide on cell-signaling regulation have been established and discussed in the literature. An interesting hypothesis has been described by Pervaiz & Clement (23). The authors consider superoxide as "oncogenic ROS" and hydrogen peroxide as "onco-suppressive ROS". They suggested that cellular state, where the ratio tilts predominantly in favor of superoxide, inhibits apoptosis and promotes cell survival. If the ratio tilts in favor of hydrogen peroxide, this creates an intracellular environment suitable for induction of apoptosis and cell death.

This hypothesis is well-grounded by numerous experimental and empirical observations on cells, animals and humans, which are summarized in several excellent review articles (21-25). First, almost all types of cancer cells are characterized by overproduction of superoxide and permanent oxidative stress due to their mitochondrial dysfunction and up-regulated NADPH-dependent oxidase complex (NOX) (21-25). In particular, the steady-state levels of superoxide are significantly higher (5-20 fold) in colon carcinoma cells (HT29, HCT116, SW480) compared to normal colon epithelial cells (FHC) and fibroblasts (33Co), as determined by increased oxidation of DHE (42). Moreover, the proliferative and metastatic potential are increasing with enhancement of superoxide level (42, 43). Second, a lot of studies indicate that hydrogen peroxide induces apoptosis in abnormal concentrations (23-25, 43, 44). Third, studies have found that overexpression of superoxide dismutase (SOD), as well as SOD-mimetics abrogate the growth and proliferation of cancerous cells in vitro and *in vivo*, which is indirect evidence about the onco-suppressive role of hydrogen peroxide (45, 46).

It seems that superoxide and hydrogen peroxide activate different signaling pathways with opposite consequences (effects) for the cells – survival or apoptosis.

However, this hypothesis does not explain the following paradox: Why several types of cancers contain high levels of SOD (especially mitochondrial Mn-SOD) and/or activities, and why in many cases, a high Mn-SOD level or activity correlates with a poor prognosis in patients (47-49)? Actually, the role of Mn-SOD during cancer progression has been studied for several decades with controversial results. The overexpression of Mn-SOD is a common event in aggressive cancers, such as: colon cancer, lung cancer, non-Hodgkin lymphoma, basal breast carcinoma (47-49). Up-regulation of Mn-SOD would lead to the accumulation of large quantities of "onco-suppressive" hydrogen peroxide in these cancers, but even that they are very resistant and invasive. Moreover, down-regulation of Mn-SOD is supposed to increase their sensitivity to anticancer therapy (49, 50). In most cases, the catalase activity does not increase and even decreases, but glutathione-peroxidase and glutathione-reductase are up-regulated in all these cancers (49, 50). This explains, at least partially, their resistance to apoptosis, because of rapid elimination of hydrogen peroxide despite the hyperactivity of Mn-SOD. The invasion of aggressive cancers is associated with very resistant (strong) mitochondrial microsatellite instability (51).

Obviously, the aggressive tumors are characterized simultaneously by several distinctive features (Figure 4): (i) overproduction of "oncogenic" superoxide that maintains mitochondrial dysfunction and genomic instability; (ii) hyperactivity of Mn-SOD, which converts superoxide into hydrogen peroxide, trying to protect the cells from oxidative stress; and (iii) overproduction of glutathione (GSH) and hyperactivity of the GSH-dependent enzymes, that eliminate the "onco-suppressive" hydrogen peroxide and thus protecting defective mitochondria from collapse. In combination, all these events provide permanent mitochondrial dysfunction and genomic instability, strong resistance and immortality of these cancer cells. It seems impossible to kill the aggressive cancers using standard chemo- or radiotherapy due to this vicious cycle, which

ensures a permanent domination of "oncogenic" ROS (as superoxide) over the "onco-suppressive" ROS (as hydrogen peroxide). The only option is to attack all molecular targets simultaneously using a combination therapy. In case of non-aggressive cancers, characterized by down-regulated Mn-SOD or down-regulated expression of GSH-dependent enzymes, it is much easier to control the situation due to rapid induction of mitochondrial collapse through suprathreshold production of ROS (regardless of the type) using standard therapeutic approaches.

Let's get back to the results of our study and in particular to the role of melatonin in the treatment of colon cancer.

The pituitary hormone – melatonin, is involved in the synchronization of the circadian rhythms of physiological functions in mammals. It is widely accepted that melatonin has a variety of anti-inflammatory, anti-neurodegenerative, and anti-cancer effects (29-31). These effects are usually attributed to its strong antioxidant properties, as well as to its involvement in the modulation of autophagy and apoptosis (29-31).

Our data demonstrate that melatonin in combination with SN38/EF24 decreases drastically the level of superoxide in the cancerous tissue, but increases the level of hydroperoxides and particularly hydrogen peroxide (Figure 3). Thus, melatonin is an antioxidant in regard to superoxide, but a prooxidant in regard to hydrogen peroxide. In this context, melatonin has to be considered as a redox-modulator than as a classical antioxidant.

We would like to emphasize a few well-known empirically and experimentally discovered facts that could provide an explanation of the anticancer effect of melatonin: (i) Melatonin is involved in the autophagy of defective mitochondria (29-31); (ii) Upon aging and stress, the sleep-wake cycle is violated and the humans usually suffer from insomnia, which is accompanied by a decrease in melatonin levels in the organism. This increases the risk of mitochondrial imbalance and genetic instability; (iii) The incidence of cancer is increasing with aging and stress.

Our hypothesis is that the anticancer effect of melatonin is attributed mainly to mitochondrial autophagy than to its antioxidant properties. The removal of the defective mitochondria and recycling of normal ones eliminates genetic instability of cells and restores the normal homeostasis. Presumably, this is the meaning of the sleep and central role of melatonin in this process.

Currently, the trigger of mitochondrial autophagy is not known, but the induction of the process might be the only option for stopping the vicious cycle of the aggressive tumors and their successful treatment. The elimination of defective mitochondria and genetic instability can be achieved by using appropriate redox-modulators in combination with conventional anticancer drugs. Melatonin is one of the most suitable candidates, because it is a natural endogenous substance, harmless to normal cells and tissues. Based on the molecular mechanisms of melatonin and other factors of circadian clock machinery, we can identify the key factor(s) that determine the choice of the cell at stress and aging – proliferation or apoptosis. This might be of outstanding significance for drug development and effective treatment of cancer.

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