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Metallothionein Colon Crypt Immuno-positivity as a Rapid *In Vivo* Essay for Drug Efficacy Studies

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Abstract. *Metallothionein immune positivity indices are considered as representative of crypt stem cell mutations. The frequency and size of MT- immunopositive foci, as well as the total number of MT-immunopositive crypts were assessed here in a short term in vivo assay. Drug efficacy was tested on early mutated crypts in colon of Balb-c mice 30 days after induction with a single dose of the mutagen dimethylhydrazine. The different drugs used (MS 275, vioxx, 5-fluorouracil, aminophylline, 5-azadeoxycytine) affected the metallothionein – immunopositive crypt frequency according to their predicted efficacy on this specific model of mouse colon carcinogenesis. This preliminary validation study of metallothionein - immunopositive crypt frequency strengthens the evidence that metallothionein immunopositivity indices could be used as short-term markers to assess the capability of different pro-drugs to counteract crypt invasion and clonal expansion of mutated stem cell progeny. This rapid in vivo test (30 days) based on metallothionein immunopositivity indices can be assayed in paraffin-fixed tissue sections and has been validated against the Glucose 6 phosphate Dehydrogenase assay. To quantify metallothionein immunopositivity indices, we devised a novel fast analysis protocol based on the Zeiss Axiovision software for image processing.*

Metallothionein (MT) crypt-restricted immunopositivity is a colonic crypt stem cell mutation marker that may be induced early and in abundance after mutagen treatment. Persistent MT overexpression within single crypts has been tested as a biomarker of colonic crypt stem cell mutation in the morphologically normal appearing mucosa. Jasani *et al*. (2) found that the frequency and time course of crypt conversion to MT immunopositivity in mice treated with dimethylhydrazine was similar to that of the Glucose 6 phosphate Dehydrogenase assay (G6PD) (2). Cook *et al*. (1) demonstrated a strong correlation between the MT and G6PD assays (r>0.9) in Balb/c mice treated with *N*-ethyl-*N*-nitrosourea (1). Stable, crypt restricted immunopositivity for MT thus correlates with an established mutation marker (G6PD) in the mouse colon. This is considered the result of a mutation affecting the expression of the *MT* gene in colonic stem cells (3). Morphologically normal-appearing mucosa from human colorectal carcinoma resection specimens and from the colons of ageing laboratory mice contain scattered single crypts whose cells exhibited uniformly increased MT immunostaining. This suggests that MT overexpression arises directly from random crypt stem cell somatic mutation, followed by colonization of the clonal unit by the mutated progeny (3-5).

The oncological relevance of MT crypt restricted immunopositivity indices (MTCRII) was also recently proved (6).

MTs are classified into a group of low-molecular weight, cysteine-rich intracellular proteins which are encoded by a family of genes containing at least ten functional isoforms in human and two isoforms in mice (1). The expression and induction of these proteins have been associated with protection from DNA damage, oxidative stress and apoptosis (1).

MT overexpression occurs frequently in human tumors, and seems correlated to a genetic signature of microsatellite instability (7).

In our study, we assessed the feasibility of using MTCRII as a rapid *in vivo* assay for drug efficacy studies during early colorectal tumorigenesis (1). The MTCRII bioassay offers the further advantage that it can be utilized on paraffin-fixed tissue sections.

Materials and Methods

Male BALB/c mice were supplied by Harlan (Indianapolis, Indiana I.N. USA). They were maintained in a limited access barrier rodent facility. Room temperature and relative humidity were continuously monitored and maintained at 22 (±2˚C) and 55% (±10%) respectively. A 12 h light-dark cycle was kept throughout the

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Table I.

Group	# Animals	Vehicle	Dose (mg/Kg)	Route/Regimen
(1) DMH	10	EDTA/NaOH	30	IP/One injection
(2) Control	5	Saline 0.9%	IP/One injection	
(3) MS275 + (DMH)	5	10% DMSO	20	IP/5 day a week
		45% PEG400		
		5% Water	30	
(4) 5 FU + (DMH)	5	Saline 0.9%	40	IP/5 day a week
			30	
(5) 5-Aza-2deoxycytidine + (DMH)	5	Saline 0.9%	1	IP/3 day a week
(6) Aminophylline + (DMH)	5	Saline 0.9%	125	IP/7 day a week
(7) Vioxx + (DMH)	10	0.5% Methylcellulose	20	Os Gavage/7 day a week
(8) Vioxx + Aminophylline + (DMH)	10		20	Os Gavage/7 day a week
			125	IP/7 day a week

Detailed description of our experimental groups. Group (1) ten animals were treated with carcinogen alone (DMH). Group (2) Control, five animals were injected *i.p.* with Saline 0.9%. Groups 3-8 were first injected with the carcinogen (DMH) and, after induction they were treated with therapeutic compounds. Group (3): DMH+MS275. Group (4) DMH+5FU. Group (5) DMH+5Aza-2deoxycytidine. Group (6) DMH+Aminophylline. Group (7) DMH+Vioxx. Group (8) DMH+Vioxx+Aminophylline. The specific vehicles, dosages and routes are listed.

experiment. Animals were supplied with a commercial diet (4RF21; Mucedola, Verona, Italy) and tap water *ad libitum*. For a detailed description of the Treatments and the Vehicles used see (Table I).

List of chemicals used: 1,2-Dimethylhydrazine dihydrochloride 40690; Aminophylline A1755; 5-Aza-2'deoxycytidine A3656; MS-275 M5568; 5-Fluorouracil F6627; Methyl cellulose M7027; Dimethyl sulfoxide 41644; (Peg.400) Poly(ethylene glycol) 81172; Methylene Blue M4159; Ethylenediaminetetraacetic acid E9884; Sodium hydroxide (Na OH) 221465; 10% Neutral Buffered Formalin HT50-1-1; all the chemicals mentioned before were from Sigma (Sigma St. Louis, MO, USA); Vioxx/Rofecoxib was a generous gift of Dr. Tony Ford-Hutchinson (Merck & Co. Rahway, NJ, USA).

At the end of the treatment period and before necropsy, mice were euthanized by compressed CO_2 gas in cylinder as indicated in the American Veterinary Medical Association Panel on Euthanasia (10).

Dissected colon samples were slit, opened and fixed flat on filter paper in 10% Neutral Buffered Formalin for 24 hrs at 4˚C. After fixation, samples were embedded in paraffin according to standard protocols (6). Colon samples used for Aberrant Crypt Foci detection were stained un-sectioned with methylene blue for 5 min at room temperature (6).

The experiment was conducted according to EU Directive EC86/609 on the protection of animals used for experimental and other scientific purposes, which was implemented by Italian Legislation with DL no. 116/92 on 19th February, 1992.

The Research Project was approved by the Italian Ministry of Health on August 25, 2003. In July 2004, Animal Procedure Statement was revised and approved by our Institutional Animal Care and Use Committee.

Experimental design. Male BALB/c mice were injected intraperitoneally (*i.p.)* in groups of 5-8 animals at 6 weeks of age with a single dose of 1,2-dimethylhydrazine (Sigma St.Luis, MO, USA) at 30 mg/kg dissolved in Tris-HCl and buffered with 1 N NaOH at pH 6.5 (Table I). It is important to note that at this stage of very early carcinogenesis, *i.e.*: 30 days after DMH injection, there is no development of tumoral masses (there is no tumor burden according to the (10) United Kingdom Coordinating Committee on Cancer Research guidelines for the welfare of animals in experimental neoplasia (10), and there is no morphological distinction in FFPE colon sections between treated and untreated mice haematoxylin and eosin (H/E) staining; the only way to detect the mutated crypts is by MTT immunostaining (1-2).

In our experimental design we had 8 groups of animals, the positive control group (group 1), the negative control group (group 2) and the antitumor test groups, groups 3 to 8 see (Table I).

Groups 1, 3-8 were injected *i.p.* with DMH. Group 1 was be exposed to carcinogen induction alone without additional treatments. Our negative control group (group 2) was injected *i.p.* with saline following the same schedule of group 1. Group 2 also received no additional treatment and it will depict the normal colon mucosa morphological landmarks. The other 6 DMH induced groups (groups 3 to 8) were treated with different antitumoral compounds starting at the wk.3 after DMH injection. For a detailed description see (Table I). The antitumoral treatments lasted other two weeks, *i.e.*: wks.3 and 4 after DMH injection (Figure 1). Our goal was to exploit the potentiality to detect an early, colon crypt restricted, anticarcinogenic activity in our short term *in vivo* mouse model. The description and/or quantization of such antitumoral activity was based on the detection of MT-positive crypt in the colon a well established biomarker for mutated colon crypts detection. (1-2). Some of the compounds we used have a well known therapeutic activity and are used in clinical practice, such as 5-flurouracil (5-FU) (8, 9), while rofecoxib-vioxx, 5-azadeoxycytine and MS275 have shown high efficacy in mouse models of colon cancer (12-14, 16-19). After treatments mice were anaesthetized and euthanized 30 days after DMH induction. A detailed necropsy was conducted on each animal and abnormalities were recorded. The colons were removed, opened, cleaned and Swiss-rolled. No macroscopic evidence of neoplastic changes were observed. Then colons were fixed in 10% neutral buffered formalin and processed to paraffin blocks (formalin fixed paraffin embedded, FFPE).

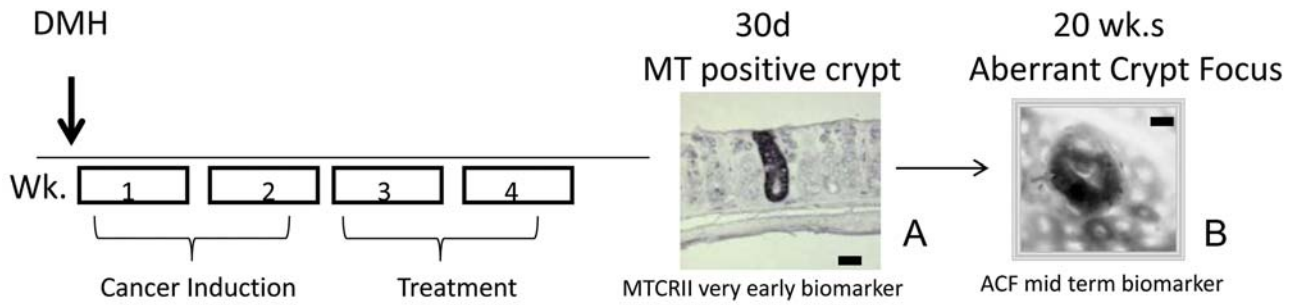

Figure 1. *Experimental design, after 2 weeks of DMH induction the therapeutic treatments were carried out during the third and fourth week. At day 30 after DMH injection the animals were sacrificed and MT-positive crypts were depicted and scored by the Zeiss Axiovision software. In A an MT-positive crypts is depicted , in B an aberrant crypt focus (ACF) is shown (magnification bar=50 μm).*

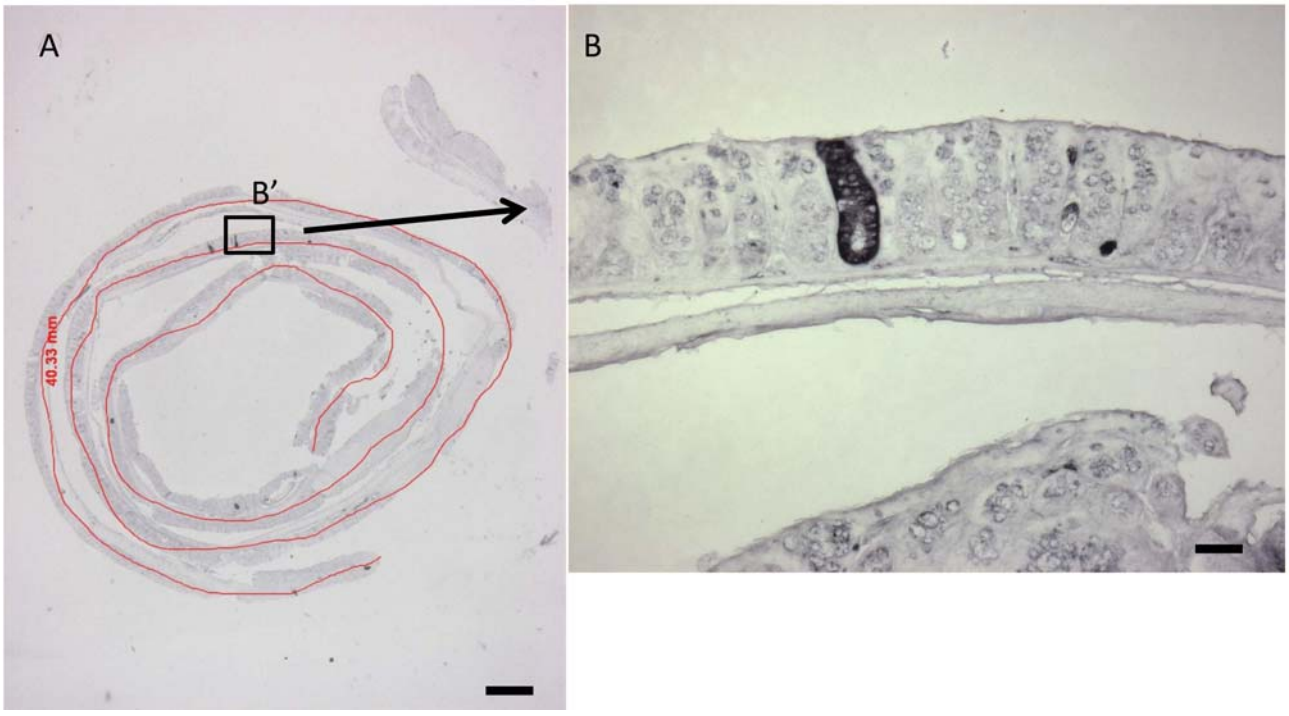

Figure 2. *A) Assessment of total crypt number. In this Figure (A) a Swiss-rolled colon of DMH-induced Balb-c mice is depicted , in sagittal orientation: 40.33 mm (total mucosa length)/50 μm (single crypt diameter)=~807 total crypt number (magnification bar=320 μm). B) High magnification of the area in A (arrow). A single MT-positive crypt is depicted. (magnification bar=50 μm).*

Time course of treatment. Groups 3 to 8 started antitumoral treatment two weeks after DMH induction (Figure 1), and for all compounds the treatment lasted two additional weeks up to the end of the 30th day (Table I). Group 3 corresponds to MS 275 treatment, Group 4 to 5-flurouracil treatment, Group 5 to 5-azadeoxycytidine treatment, Group 6 to Aminophylline treatment. Group 7 to rofecoxib-vioxx treatment, and Group 8 to rofecoxib-vioxx/aminophylline treatment (Table I).

At the dosages used, there were no mortalities as a result of the study, and clinical signs observed were minimal and fell within those described in literature (12-18). The average reduction of body weight did not exceed 10% of that recorded before the beginning of experiments.

Immunohistochemistry. Immunohistochemistry was performed essentially as described by Lazzaro *et al*. (11). Paraffin sections (5 μm) through the Swiss-rolled colon were cut at different levels and 5 μm sections were collected 100 μm apart and stained using a standard indirect immunoperoxidase technique for MT. Sections were cleared in xylol and re-hydrated, the unmasking procedure was carried out by immersing the samples in DAKO antigen retrieval solution 10x (DAKO Cytomation, Carpinteria, CA USA) at 99˚C for 40 min. After rinsing in PBS, the sections were covered with blocking solution, 15 μl goat serum in 1 ml PBS 0.1% triton (PBS TRN), for 20 min at room temperature. The anti-MT primary antibody (E9, MO639, isotype: IgG1; DAKO Ltd, Ely,

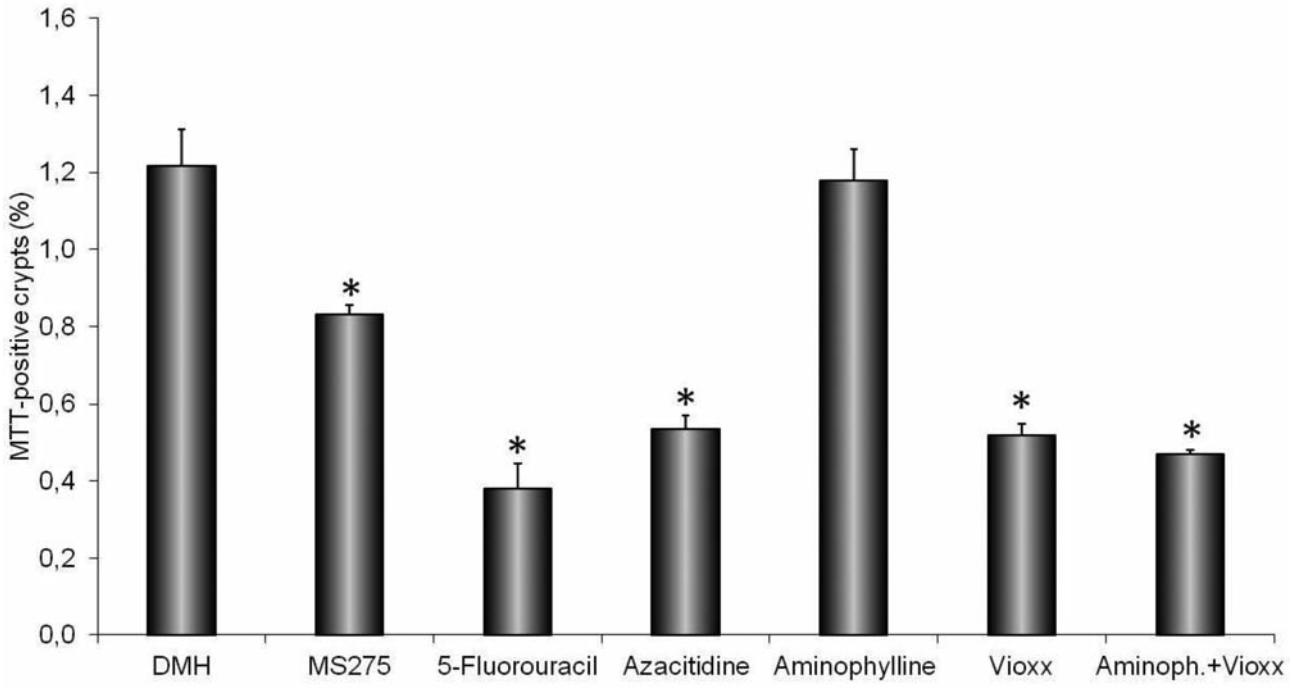

Figure 3. *Percentage of metallothyonein-positive crypts, after treatments. Each column corresponds to 5 animals. (*p<0.001 Student's t-Test).*

Cambridgeshire, UK) was biotinilated by Zenon Biotin-XX Kit (Z25052; Molecular Probes Grand Island, NY 14072 USA). This antibody recognizes both isoforms of the MT protein and is directed against a highly conserved epitope. Without additionally rinsing after blocking, the slides were covered with the anti-MT primary antibody and incubated over night at 4˚C.Three washing steps followed with PBS TRN, PBS 3% H_2O_2 (3 min) and PBS, respectively. Alternatively for Acetylated Histone H3 detection, sections of 5 μm from five animals of Group 1, Group 2 and Group 3, were stained with a primary antibody specific for the acetylated histone H3 (lysine acetylated) (06-599 Upstate, Lake Placid, MA USA) diluted 1:500. Then sections were rinsed in PBS TRN and PBS and incubated with anti-rabbit antibody for 30 min at room temperature. For signal amplification analysis, sections were incubated with ABC Complex (VECTASTAIN ABC Kit, PK 6100, from VECTOR Burlingame, CA USA) for 30 min at room temperature, then rinsed in PBS and stained with the diaminobenzidine (Peroxidase substrate kit, SK 4100, from VECTOR Burlingame, CA USA) for 1 min.

Sections were dehydrated and mounted with Entellan (OB 043984 Merck KGaA. Darmstadt Germany). A Musachi (MSI1)-specific rabbit monoclonal from Abcam (ab52865) (Abcam plc Cambridge UK) was also used to detect the stem niche in the colon crypts. The antibody was diluted 1:200 and revealed using a secondary biotinylated Ab. For signal amplification analysis, sections were incubated with ABC Complex (VECTASTAIN ABC Kit, PK 6100, VECTOR Burlingame, CA USA).

Percentage of MT positive crypts. To determine the percentage of MT-positive crypts (MTCRII) we developed a new rapid procedure that is less time consuming than those described in literature (1-2). Quantification is based on the Zeiss Axiovision (Carl Zeiss Jena, Germany) software for image acquisition. We acquired an image of the entire Swiss-rolled, sectioned mucosa at low magnification (x 0.6) with the use of a Zeiss Stemi SV 11 Stereomicroscope and a Zeiss Axiocam HRc CCD camera.

The total length of the section was measured using Zeiss Axiovision software. Considering that the average diameter of a crypt is 50 μm, we were able to rapidly assess the total number of crypts per section and immediately calculate the MTCRII (Figure 2A).

To ensure the error of estimation was not exceedingly great, we scored the total number of crypts by counting them for each section analysed with the Zeiss Axiovision Software. The mean relative Error (MRE) was calculated as the average of the relative error: $MRE=\Sigma^{n}_{i}=1$ relative $error_n/_n$, where relative error is the ratio: absolut error / calculated value of error.

We also calculated the standard deviation (S.D.) on the MRE. On 46 colons scored we found that the MRE was approximately 4%, and only in two cases was it there a significant difference between the observed and the assessed values (data not shown). We thus concluded that the scoring of the total number of crypts could be carried out by the software analysis without committing significant errors of evaluation. The black MT-positive crypts were counted and the percentage of MT-positive crypts (MTCRII) was calculated as the ratio of the number of MT-positive crypts and total crypts counted (data not shown).

Results

By using the Zeiss Axiovision software and by counting the crypts we scored the percentage of MT-positive crypts and/or metallothionein crypt-restricted immuno-positivity indices (MTCRII). MTCRII was recently described as a reliable very

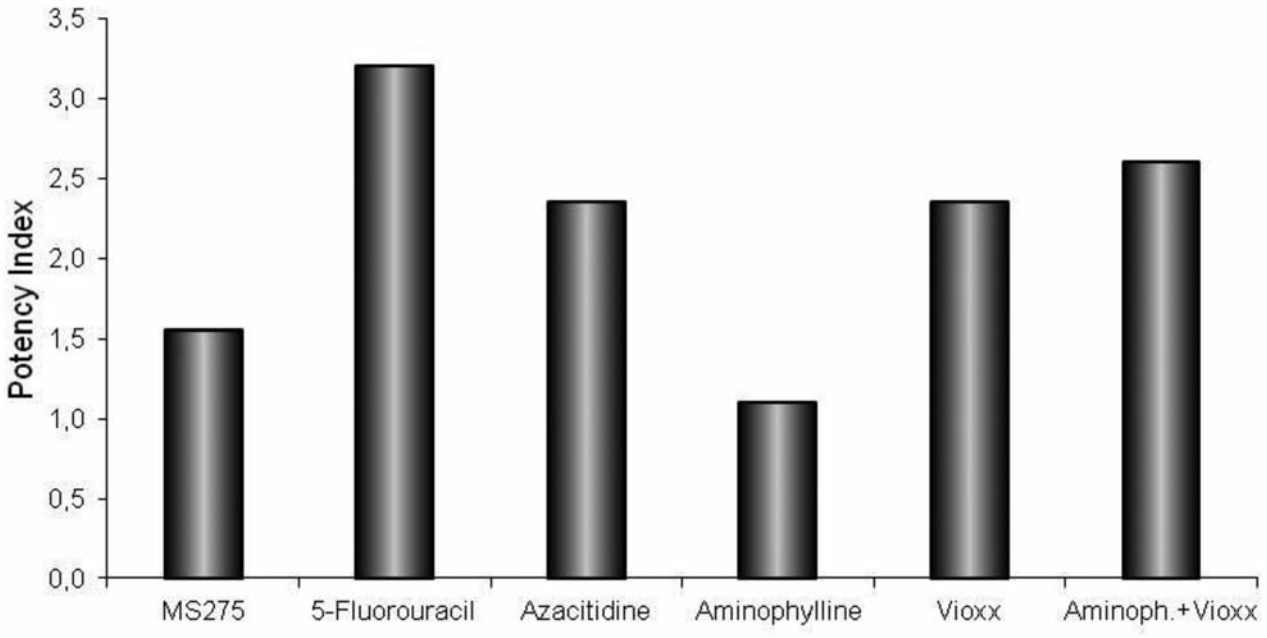

Figure 4. *Potency index indicates the fold-reduction in the carcinogenesis end-point due to the agent. A 3-fold reduction in percentage of MT- positive crypts for 5-FU, azacitidne and vioxx was induced by treatments. As expected aminophylline + vioxx potency index is identical to the value of vioxx alone. MS275 is showing a much lower potency index (~50 percent less) compared to 5-FU, azacitidine and vioxx.*

early murine biomarker relevant for colon cancer tumorigenesis (6). Donnely *et al*. (6) described a direct correlation between metallothionein crypt-restricted immuno-positivity and Aberrant crypt foci (ACF) (Figure 1). ACF are the earliest preneoplastic lesions detectable by morphological means (6). Therefore our goal was to prove that MTCRII can be used as a predictive very early colon cancer biomarker, which is detectable far in advance than Aberrant crypt foci (Figure 1). MTCRII are detectable in a morphologically normal mucosa, depicting mutated crypts which are morphologically identical to normal ones (1-2, 6) (Figure 2 A and B), and also expressed our data as a potency index (P.I.).

P.I. is the ratio of MT-positivity and the control/treated mice and indicates the fold change in a carcinogenesis endpoint related to the agent used for treatment. According to this definition, any P.I. > 1 indicates a potential level of efficacy induced by the treatment as a variation of the ratio between the value of MTCRI in the control and treated samples. As shown in Figure 3, 1.2% of MT-positive crypts in DMH-induced colons which did not receive any treatment. This value is close to the percentage of mutated crypts in aminophylline treated samples. Aminophylline as expected, did not affect the MTCRII in a statistically significant manner (P.I.=1).

By contrast, the 5-FU, vioxx, aminophylline + vioxx, 5-azacitidine and MS275 treatments produced different but statistically significant reductions of the MTCRII in the different groups. 5-FU was the most effective, inducing a 66% reduction of MTCRII, with a P.I of 3 (Figures 3-4). Vioxx, 5-azacitidine and vioxx plus aminophylline treatment gave similar values, inducing a 50% reduction of MTCRII, P.I. of 2-2.5. MS275 induced an appreciable reduction of 33% in the number of mutated crypts. Comparing the vioxx (P.I.=2,25) and vioxx + aminophylline (P.I.=2.5) groups it would seem that aminophylline induced a slight increase in the efficacy of the vioxx treatment alone.

The activity of MS275 on colon samples was assessed by immunohistochemistry. Five colon samples from animals in groups 1-3 were immune-stained with acetylated histone H3-specific antibody. As shown in Figure 5, the HDACs inhibition restored the nuclear staining specific to acetylated histone H3 in the group 3(DMH + MS275) (Figure 5 C) compared to the DMH induced colon group 1 (Figure 5 B). The staining is localized at a level close to the signal depicted in the colon crypts from the saline-treated animals of group 2 (Figure 5 A).

MSI1 detection in crypts. In order to further validate MTCRII we used MSI1 as a colon crypt stem cell marker. As shown in Figure 6A an MSI1-specific signal was restricted in the lower third of the crypt, where the stem cell niche resides, in the vehicle treated samples (group 2) (Figure 6A arrows). Crypts are made by 3 different morpho-functional segments, arrows are indicating the lower third of the crypt where the stem cells

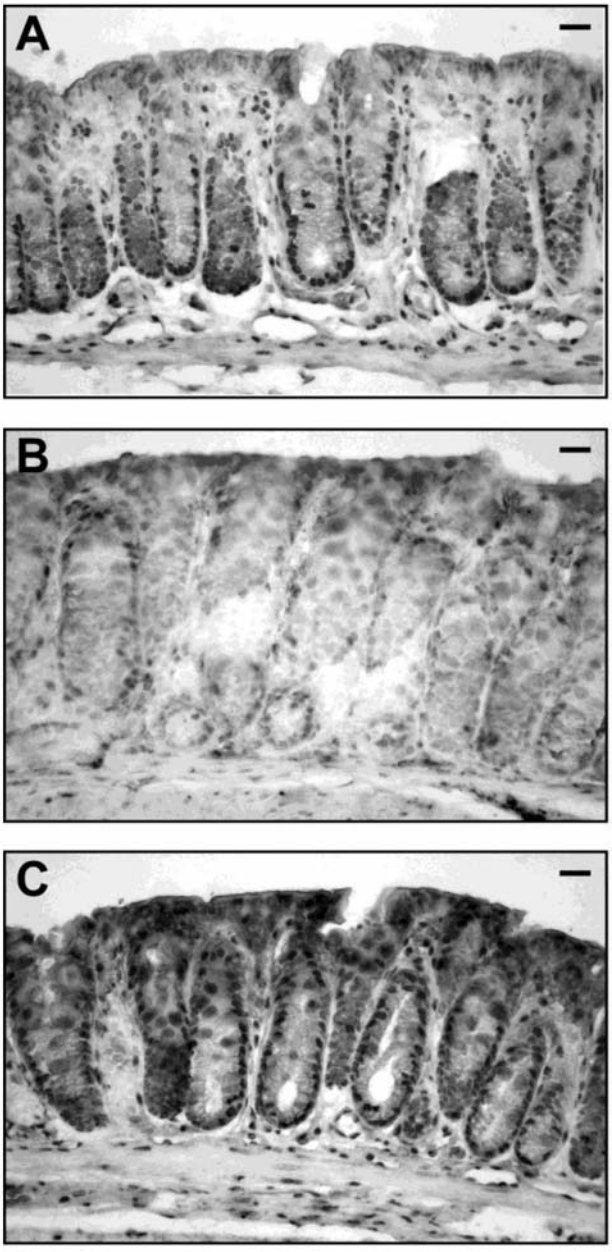

Figure 5. *Immunohistochemistry for acetyl histone H3. 5A: Sample from a control mouse treated only with Vehicle; the staining is nuclear and widely distributed. 5B: Sample from DMH treated mouse, acetyl histone H3 specific nuclear staining was highly reduced. 5C: Sample from a MS275-treated mouse, pretreated with DMH; nuclear staining is restored and similar to that shown in A. (magnification bar=50 μm).*

niche reside. Normal proliferating cells are restricted in the lower third of the crypt (1-2, 11).

In carcinogen-treated samples (group 1) the signal is upper-shifted in the mid- and upper-third of the crypts (the non proliferative compartments of the crypt). This is the description of a dysfunctional proliferation pattern, that is possibly a consequence of mutated stem cell colonization of the entire crypt (Figure 6B, arrows).

In 5-FU-treated samples (group 4) MSI1 signal is undetectable in any segments of the crypt (Figure 6C, arrows) (arrows are depicting the morpho-functional units of a crypt). In MS275 treated samples (group 3), 5-aza-cytidine (group 5) and vioxx treated samples (group 7), the MSI1 specific signals is again restricted to the lower third of the crypt (Figures 6D-F, arrows). The Figure shows a sample 6G aminophylline-treated group (group 6). The MSI1-specific signal is upper-shifted in the mid and upper third of the crypts (arrows). Figure 6H is a scheme depicting the distribution of proliferative and differentiative compartments of the crypt.

Discussion

The aim of the present study was to validate the MTCRII in the Balb-c/DMH mouse model of early colon tumorigenesis as a suitable *in vivo* biomarker for drug efficacy studies on mutated stem cells. To achieve this goal we used four different molecules (5-FU, 5-aza-deoxycytine, vioxx, MS275 and aminophylline) which have differing antitumoral activity in various *in vitro* and *in vivo* tumor models (7-8, 12-18, 20-21, 23-25, 31).

5-FU was considered our benchmark due to its wide application as an antitumoral agent for a variety of solid tumors in human therapy and in DMH colon carcinogenesis animal models (13-14). Vioxx (rofecoxib), a selective (COX-2) inhibitor, was also active in this model as described by Oshima *et al.* (8) and Noguera Aguilar *et al.* (9). 5-aza-deoxycytine has been successfully used on DMH-treated animals (16-17). MS275 is an HDAC inhibitor which can potentially induce an epigenetic reprogramming of the transformed cells, and its antitumoral efficacy on xenografts of human colon carcinoma cell lines has been reported (18). We also decided to use aminophylline, which increases the levels of cAMP, as it is well known that high intracellular quantities of cAMP can destroy cancer cells *in vitro* (19) and substances that augment cAMP such as forskolin, 8-bromo-cAMP, 8-chloro-cAMP, monobutyryl or dibutyryl, cannot be used as anticancer drugs due to their cytotoxicity. In contrast, blockers of phosphodieterases (PDE), such as theophylline and aminophylline, (which increase intracellular cAMP), are routinely used as anti-asthma drugs (19). Aminophylline and theophylline are methylxanthines which have been therapeutically used in experimental models of human B-cell leukemia (20) and, more recently, in human clinical studies for B-cell leukemia therapy (21). It has been further reported that theophylline and aminophylline could affect lung cancer survival and act with gemcitabine and cisplatin to induce apoptosis in the lung cancer cell line (H1299) (22) and in a variety of carcinoma cell lines (19, 22). In the rodent colon tumors induced with DMH, the concentration of cAMP is half the one found in the normal mucosa, and immediate increases in the cAMP-PDE

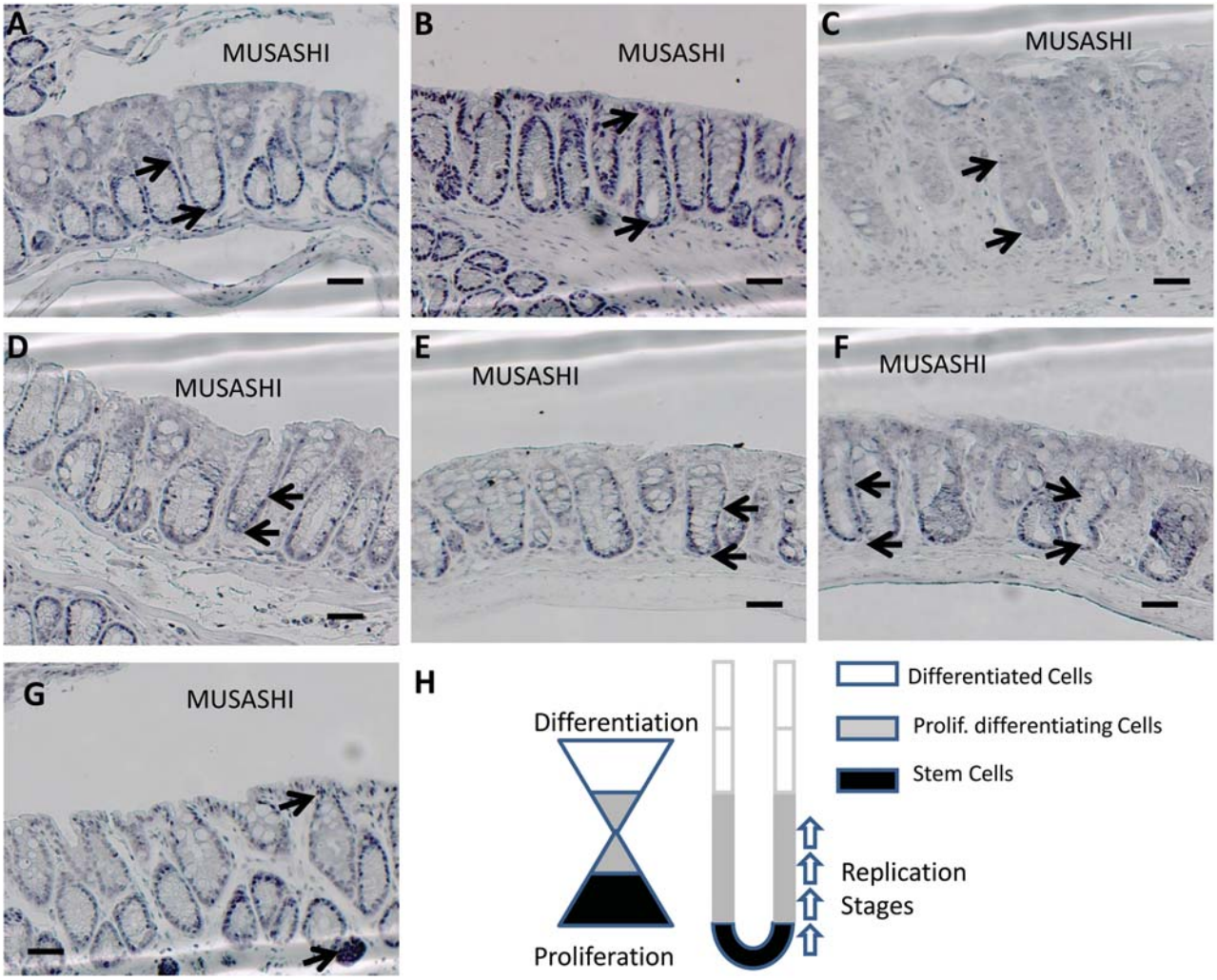

Figure 6. *Musashi MSI1 expression pattern is depicted in colon crypt of mice after different treatments. A: Sample from a control mouse treated only with Vehicle, MSI1 is restricted to the lower third of the crypt (arrows). B: Sample from a mouse treated only with DMH; MSI1 expression is upper shifted in all the functional segments of the crypt (arrows). C: sample from a 5-FU treated mouse pretreated with DMH; MSI1 is almost undetectable in the all crypt segments (arrows). D-F: Samples from mice pretreated with DMH and treated with MS-275 (D), (5-AZA) (E) and vioxx (F) respectively. After treatment, MSI1 signal is restricted to the lower third of the crypt D-F (arrows). G: Sample from a mouse pretreated with DMH and treated with aminophylline; MSI1 expression is upper shifted in all the functional segments of the crypt (arrows). H: (Schematic representation) Distribution of the proliferative and differentiative compartments of the crypt (magnification bar=70 μm).*

activities were found in the tissues after a single exposure to DMH (23, 24). In previous studies on human colon cancer samples, it was shown that methylxantine failed to significantly modify cAMP levels in the neoplastic mucosa as compared to adjacent normal mucosa (25). Vioxx clearly prevents DMH-induced colon carcinogenesis in rodents, with a reduction of tumoral colonic percentage in adenocarcinomas and of tumoral COX-2 expression (9) Considering that aminophylline was not going to be active in our model, we decided to further test this hypotesis combining aminophyllin and Vioxx treatments. These two drugs, vioxx and aminophyllin, apparently have opposite effecs on cAMP intracellular concentration, therefore they cannot synergize since vioxx decreased the cAMP intracellular concentration, by inhibiting prostaglandin E2 synthesis (26, 27).

As expected 5-FU was the most effective in reducing the percentage of MT-positive crypts in DMH-treated animals, with a 60% reduction of the MTCRII and a P.I. of 3.1. It is worth noting that the proliferative uppershift in mutated colon crypts is one of the first signs of progressive colonization by a mutated stem cell progeny in humans and in rodents (3).

During colon development and in adult life, COXs play a pivotal role in the maintenance of the intestinal stem niche (29). The over-expression of COX-2 has been observed in human colon tumors (Hlavaty *et al*., 2004) (28) and in

rodent tumors induced by colon specific chemical carcinogens such DMH (9). COX-2 is also overexpressed in transgenic mouse models reproducing mutations in the human adenomatous polyposis gene (8). In both mouse and human models COX-2 inhibitors such as vioxx have been found to be extremely effective in reducing the number and size of intestinal and colonic polyps (8, 9). It was therefore expected that a potent inhibition of mutated crypt expansion would be elicited by vioxx treatment, with a 50% reduction of MTCRI and a P.I. of 3.

The importance of altered DNA methylation and its implication in cancer pathogenesis has been reported (4, 30), and DNA methyltransferase 1 (Dnmt1) is overexpressed in colon tumor cells as compared to normal cells (31). The inhibition of Dnmt1 activity by 5-aza-deoxycytine tends to restore the transformed phenotype to normal in different colon cancer tumor cell lines (32, 33). 5-aza-deoxycytine treatment affects aberrant crypt formation in rat colon induced by DMH (16). We were also able to confirm this in our early carcinogenesis model; 5-aza-2-deoxycytidine induces a strong reduction of the (MTCRII) with a P.I. of 2.3.

Synthetic benzamide derivatives were investigated for their ability to inhibit HDACs, and among these benzamide derivatives, MS275, has proven antitumoral efficacy(18). MS275 inhibited partially purified human HDACs and induced hyperacetylation of nuclear histones in several tumor cell lines. Its behavior resembles the one of other HDACs inhibitors, such as sodium butyrate and trichostatin A; MS275 induced p21 (WAF1/CIP1) and gelsolin, and interfered with the cell cycle distribution, reduced the proportion of S-phase cells, and increased those of the G_1-phase. In our model, MS275 led to a 33% reduction of the total of MTCRII with a P.I. of 1.5. Our observation suggests that the therapeutic potential of MS275 were highlighted in our model, as expected, since this class of anticancer molecule has found an effective application in experimental oncology and also in clinical trials as single modalities and in combination with chemotherapeutic agents (17-18).

Theophylline and aminophylline are PDE inhibitors, which possibly exert their anticancer activity through blocking cAMP formation, through adenylate cyclase activity. Inhibition leads to various effects on cellular biology, at apoptotic, cell growth and cell motility levels. MDM2 counteracts the p53 apoptotic action and its down regulation, induced by methylxanthine, restores normal apoptotic events (34, 35). High intracellular concentration of cAMP induced by methylxanthine down-regulates RAS, MAPK signaling, inducing cell arrest and cytoskeleton reorganization (35).

Aminophylline was used at the highest tolerated dosage, but no significant reduction of the MTCRII percentage was observed. As mentioned above, aminophylline was not active alone, and the combined treatment with vioxx did not induce any appreciable increase compared to vioxx alone.

In conclusion it is generally accepted that colonic epithelial stem cells are the principal cell type at risk of accumulating progressive somatic mutations, leading to cancer progression and carcinoma (3, 37). The MTCRII reflects the colonic crypt stem cell mutations that are induced early and in abundance after mutagen treatment, and has recently been shown to correlate with formation of aberrant crypt foci formation 20 weeks after the initiation of treatment (6). Aberrant crypt foci are the first morphologically detectable colonic precancerous lesions (38). We observed through our study that modifications of the MTCRII in our model by therapeutic agents are mostly in line with their predicted efficacy, on the basis of their different mechanisms, the variation of efficacy on the MTCRII was partly predictable. Since we were able to consistently reproduce such variations, confirming the putative efficacy and/or inefficacy of these molecules in our model, we feel confident that our study is a preliminary validation of MTCRII as a reliable short-term biomarker for the *in vivo* screening of anticancer molecules.

It has recently been demonstrated that once crypts are wholly-mutated, there is subsequent clone expansion by crypt fission, the mechanism through which mutations spread in the normal human colon (39). MTCRII could therefore prove to be a useful tool to explore the efficacy of antitumoral agents on early tumorigenic events, and the spread and/or expansion of mutated stem cell populations in the colonic epithelium. MTCRII can trace the various stages of the otherwise morphologically invisible genetic progression and niche expansion of mutated stem cell (5, 37). Drugs that target only end-stage or differentiated cells may have immediate palliative effect, but only by targeting stem cells are we likely to obtain long-term curative effects. We have described a simple and reliable model that allows the *in vivo* study of modifications induced by antitumoral molecules on the early stages of colon crypt colonization by mutated stem cells.

We further validated our procedure using the mRNA translational control protein, MSI1, a functional biomarker of the stem cell niche in the lower third of the crypt. We clearly showed that the different drugs can affect the stem cell niche, as demonstrated by MSI1 signal distribution in the colon crypt.

MSI1 plays a critical role in promoting physiological stem cell self-renewal and has been implicated in the development and progression of neural, colon, breast and hematopoietic cancers (40). Recently, it has been shown that Adenomatous polyposis coli (APC) MSI1 interactions maintain homeostatic balance in the intestinal epithelium (40). APC is a tumor suppressor gene, which is playing a major role in colon cancer repression in animal models and human patients (11, 40).

The recent observation of Field Defects in Solid Tumors (11, 42) is lending credence to the hypothesis that in such tumors both resting and proliferative compartments are

generated by the same subset of cancer stem cells (40-46). Solid tumor field-defects are well documented in colon cancer (42). They are the result of progressive accumulation of mutations in restricted population of tumor initiating cells (11, 37-43). Multiple mutations can be accumulated only in subsets of proliferating cells called stem cells (41-46). Normal stem cells and cancer stem cells share the same potentiality of proliferative clonal expansion (43-46). The field-defects observation in a solid tumor mass and particularly in colon rectal cancer (CRC) (11, 42-46) is strongly suggesting that heterogeneous sub populations of mutated cancer stem cells can simultaneously exist in the same solid tumor (41-46). These subpopulations of cancer stem cells are hierarchically organized; each clone has different potentiality and they compete for the ultimate phenotypic imprint of the tumor mass (41-46). Ultimately the solid tumor's clinical fate depends on the constitutive mutations accumulated in the cancer stem cell clone that prevails in the solid tumor mass colonization (42-46). The most accredited hypothesis is that only a restricted subpopulation of cells with highly proliferative capability, named stem cells, can progressively accumulate in their progeny a number of mutations, both genetic and epigenetic, sufficient to generate a clone of tumor-initiating cells (42-46). Tumor-initiating cells represent a small percentage of the tumor mass (41-44). Only therapeutic interventions capable of targeting tumor-initiating and cancer stem cells can be successfully committed to eradicating cancer formation (41-46). In this complex scenario new methods which enable the discrimination between different clones of cancer stem cells or tumor-initiating cells would be vital for the definition and discovery of new classes of antitumor compounds, small molecules and or biologicals (41-46).

Tumors arising from the intestinal epithelium are likely to be controlled by similar mechanisms of development (41, 44-46). The MTCRII is a very well-established marker for identifying mutated colon stem cells and our model for the first time has shown it can be successfully used for efficacy studies of anticancer therapies on solid tumor during mutated cell crypt colonization and or expansion (3, 41-46). To the best of our knowledge, our study describes the first such use of this methodology *in vivo*.

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