Cancer Science
Japanese Cancer Association JCA
Open Access

Dual preventive benefits of iron elimination by desferal in asbestos-induced mesothelial carcinogenesis

Li Jiang, Shan-Hwu Chew, Kosuke Nakamura, Yuuki Ohara, Shinya Akatsuka and Shinya Toyokuni

Department of Pathology and Biological Responses, Nagoya University Graduate School of Medicine, Nagoya, Japan

Key words
Asbestos, chelator, desferal, iron, mesothelioma

Correspondence
Shinya Toyokuni, Department of Pathology and Biological Responses, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan.
Tel: +81-52-744-2086; Fax: +81-52-744-2091;
E-mail: toyokuni@med.nagoya-u.ac.jp

Funding Information:
National Cancer Center; Ministry of Education, Culture, Sports, Science and Technology of Japan; Yasuda Medical Foundation

Received February 11, 2016; Revised March 31, 2016; Accepted April 9, 2016

Cancer Sci **107** (2016) 908–915

doi: 10.1111/cas.12947

Asbestos-induced mesothelial carcinogenesis is currently a profound social issue due to its extremely long incubation period and high mortality rate. Therefore, procedures to prevent malignant mesothelioma in people already exposed to asbestos are important. In previous experiments, we established an asbestos-induced rat peritoneal mesothelioma model, which revealed that local iron overload is a major cause of pathogenesis and that the induced genetic alterations are similar to human counterparts. Furthermore, we showed that oral administration of deferasirox modified the histology from sarcomatoid to the more favorable epithelioid subtype. Here, we used i.p. administration of desferal to evaluate its effects on asbestos-induced peritoneal inflammation and iron deposition, as well as oxidative stress. Nitrilotriacetate was used to promote an iron-catalyzed Fenton reaction as a positive control. Desferal significantly decreased peritoneal fibrosis, iron deposition, and nuclear 8-hydroxy-2′-deoxyguanosine levels in mesothelial cells, whereas nitrilotriacetate significantly increased all of them. Desferal was more effective in rat peritoneal mesothelial cells to counteract asbestos-induced cytotoxicity than in murine macrophages (RAW264.7). Furthermore, rat sarcomatoid mesothelioma cells were more dependent on iron for proliferation than rat peritoneal mesothelial cells. Because inflammogenicity of a fiber is proportionally associated with subsequent mesothelial carcinogenesis, iron elimination from the mesothelial environment can confer dual merits for preventing asbestos-induced mesothelial carcinogenesis by suppressing inflammation and mesothelial proliferation simultaneously.

Asbestos is a natural silicate mineral. During the last century, huge amounts have been used in industry due to its excellent characteristics of resistance to heat, acid, and friction with flexibility and economic merits as a material.[1,2] However, fine asbestos fibers are present in the air of mines, factories, or construction workplaces and can pass through the respiratory system to the lung parenchyma and, finally, after decades, to the parietal pleura of affected personnel, in the event that proper preventive strategies were not taken. In the 1960s, epidemiologists already recognized the association between asbestos exposure and malignant mesothelioma (MM).[2] Despite a variety of efforts, MM remains among those tumors with the poorest prognosis when diagnosed. Its incidence is increasing worldwide, but it is resistant to currently available therapeutic methods.[3–5] Therefore, it is necessary to find efficient strategies to prevent, slow, and cure MM.

We were therefore prompted to elucidate the carcinogenic mechanism of asbestos-induced MM. Our previous preclinical studies revealed that the pathogenesis of asbestos-induced mesothelial carcinogenesis is closely associated with local iron overload,[6] which promotes the Fenton reaction and leads to oxidative DNA damage and, finally, to a variety of mutations. Iron overload has generally been associated with carcinogenesis.[7–10] Simple i.p. injection of each one of the three kinds of commercially used asbestos (chrysotile, crocidolite, and amosite) efficiently causes peritoneal MM in rats, with extremely similar genomic alterations and prominent iron deposition.[6] Irrespective of the types of asbestos, postadministration of nitrilotriacetate (NTA; iron chelating agent to promote the Fenton reaction)[11,12] significantly shortened the periods of carcinogenesis, confirming the major role of iron in this process.[13,14] In the case of chrysotile, which does not include iron in itself, its hemolytic activity and the subsequent adsorption of hemoglobin were important,[15] in addition to the general affinity of asbestos to histones.[16] We also reported that i.p. injection of iron saccharate with NTA in rats induced MM and homozygous deletion of the *Cdkn2a/2b* tumor suppressor gene, supporting the hypothesis that iron overload is responsible for asbestos-induced mesothelial carcinogenesis.[17]

Based on these findings, we recently used an oral iron chelating agent, deferasirox, to prevent MM in a rat peritoneal MM model, and we found that this preventive treatment leads to a favorable histological transition of MM from the sarcomatoid subtype to the epithelioid subtype.[18] Here we used a classic iron-chelating agent, desferal (desferrioxamine), to evaluate whether this treatment is useful for the prevention of

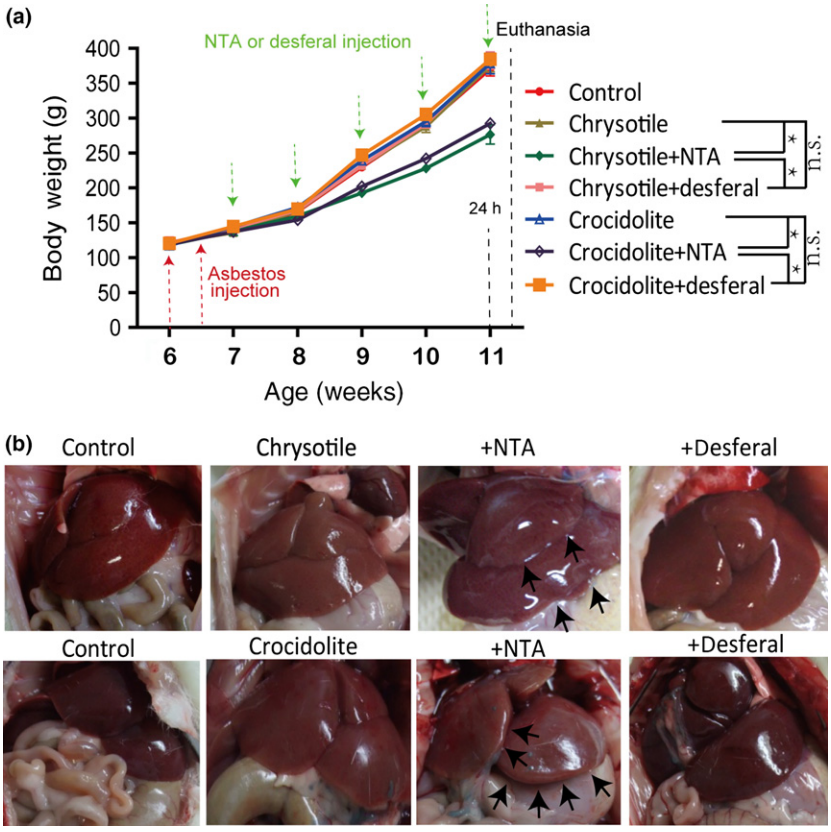


Fig. 1. Body weights and macroscopic view of peritoneal cavity after asbestos injection in rats in the presence or absence of iron chelators. (a) Body weight changes. (b) Macroscopic peritoneal appearance 5 weeks after injection of asbestos. Intraperitoneal injection of asbestos caused chronic inflammation and fibrosis in the peritoneal cavity, which was aggravated by nitrilotriacetate (NTA) (arrows show deformation of edge of hepatic lobes, indicating severe serosal fibrosis) and ameliorated by desferal. n.s., statistically not significant (means ± SEM) $*P < 0.05$.

MM after exposure to asbestos using rat and cell culture models.

Materials and Methods

Chemicals. Desferal was purchased from Novartis Pharma (Basel, Switzerland), and NTA disodium salt was purchased from Nacalai Tesque (Kyoto, Japan). Holotransferrin (iron-saturated transferrin, human) was from Sigma (St. Louis, MO, USA).

Asbestos and animals. Chrysotile and crocidolite fibers from the Union for International Cancer Control (Geneva, Switzerland) were suspended in physiological saline (5 mg/mL) and autoclaved prior to injection into rats. The suspension of asbestos was sonicated for 30 min immediately before use to prevent the aggregation of the fibers. We used another iron chelator, NTA,[19] to promote the Fenton reaction[12,20] with the dose previously described[6,21] for a comparison with the effects of desferal. Thirty-five 6-week-old specific pathogen-free male Wistar rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan) were divided into the following seven groups of five animals each: saline treatment of the same volume as asbestos suspension, as controls; chrysotile treatment; chrysotile and NTA treatment; chrysotile and desferal treatment; crocidolite treatment; crocidolite and NTA treatment; and crocidolite and desferal treatment. The asbestos-treated groups received two i.p. injections of 5 mg chrysotile or crocidolite (total 10 mg) during the first week. Nitrilotriacetate (80 mg/kg) or desferal (100 mg/kg) were injected i.p. once a week for a total of 5 weeks, starting from the second week after the asbestos injection (Fig. 1a). The animals were euthanized 24 h after the last injection of NTA/desferal or at the corresponding time, and autopsies were carried out. The animal experiment committee of Nagoya University Graduate School of Medicine (Nagoya, Japan) approved this experiment.

Histological and immunohistochemical analyses. The tissues were fixed with 10% buffered neutral formalin solution. After dehydration, tissue specimens were subjected to paraffin embedding, cut to a 3-μm thickness and stained with HE, Masson trichrome for fibrosis, or Perl's iron staining. For immunohistochemical analyses, the avidin–biotin complex method with peroxidase was used, and quantitative analyses were undertaken both for areas and integrated density as described[22] except for ImageJ was used (https://imagej.nih.gov/ij/) as a software (previously NIH image was used).

Antibodies. An anti-8-hydroxy-2′-deoxyguanosine (8-OHdG) mouse mAb N45.1 (Nikken Seil, Fukuroi, Japan) was used to evaluate oxidative stress at the cellular level with immunohistochemistry.[22]

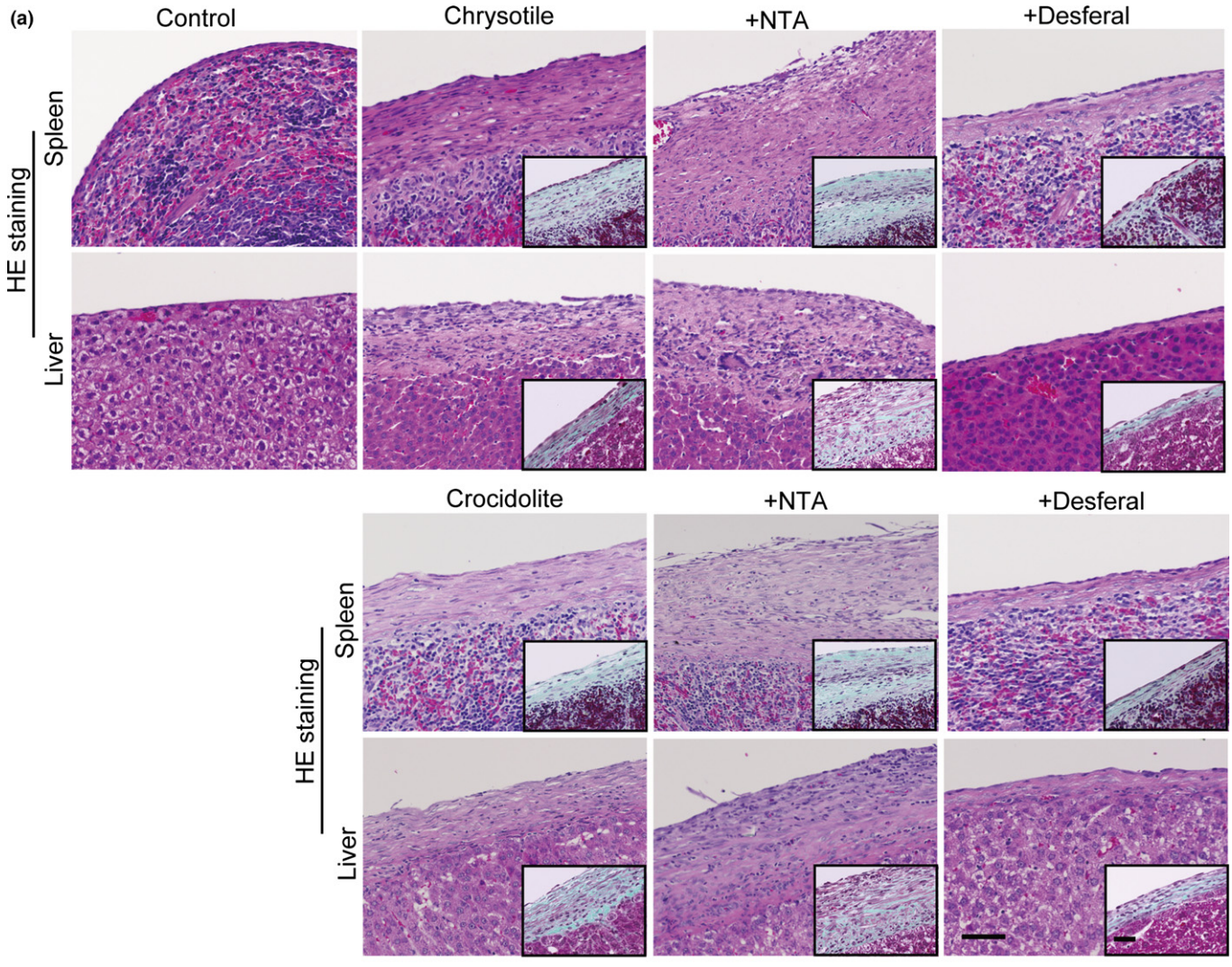
Fibrosis index. The fibrosis index was determined as previously described.[23]

Serum ferritin assay. Commercial kits were used to determine the levels of rat serum ferritin (PRA011; Mitsubishi Chemical Safety Institute Ltd, Uto, Japan).

Cell culture. Rat peritoneal mesothelial cells (RPMC) and murine macrophages (RAW264.7) were cultured in RPMI-1640 medium containing 10% FBS as previously described.[23] Malignant mesothelioma cell lines from rats were established [24] and cultured as described.[25]

Viable cell evaluation. Viable cells were either counted by Trypan blue dye exclusion or quantified with an MTT assay (Sigma). The cells were counted 48 h after exposure to desferal with simultaneous pretreatment with different concentrations of holotransferrin using Trypan blue and expressed as percentages of control cell counts.

Statistics. Data are shown as the means ± SEM. We used an unpaired *t*-test, which was modified for unequal variances



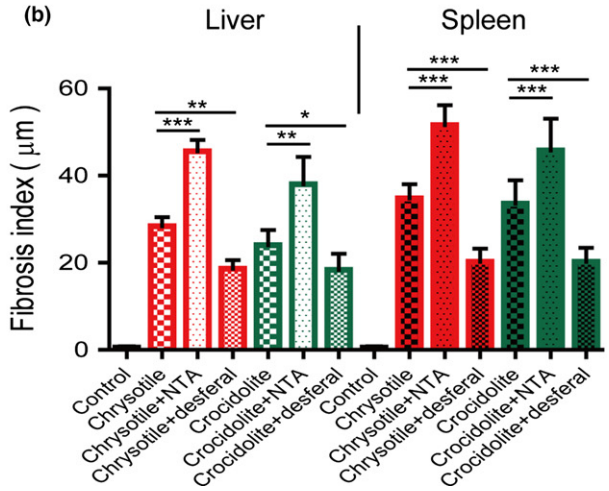


Fig. 2. Microscopic findings of serosal surface of the spleen and liver in rats 5 weeks after asbestos injection in the presence or absence of iron chelators and fibrosis index. (a) Typical observation with HE and Masson trichrome (insets) staining (bar = 50 μm). (b) Fibrosis index was calculated by measuring the distance from the peritoneal surface to the parenchyma of spleen or liver (means ± SEM). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. NTA, nitrilotriacetate.

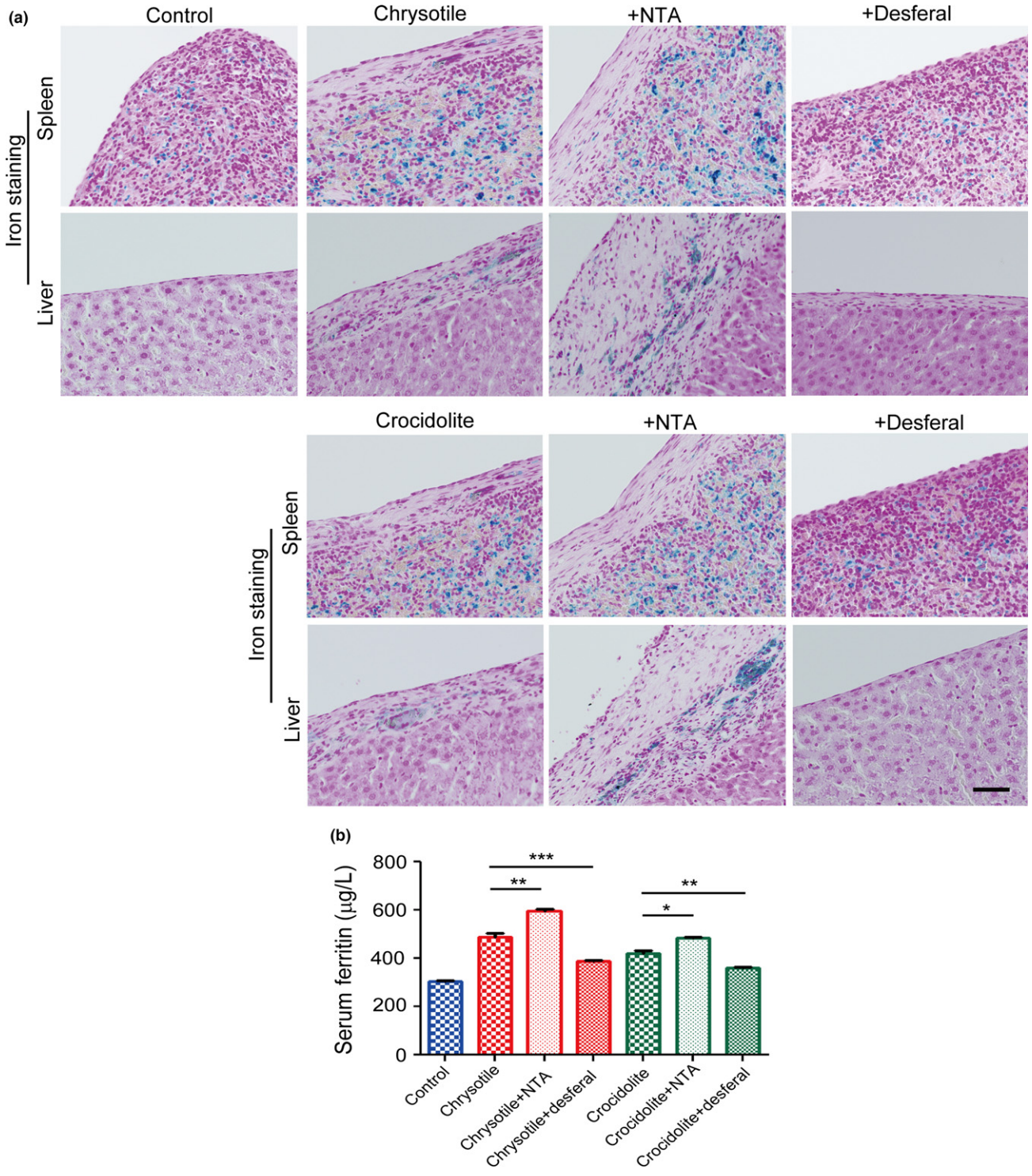


Fig. 3. Quantitation of iron deposition on the surface of the spleen and liver and serum ferritin levels in rats 5 weeks after asbestos injection. (a) Iron deposition (hemosiderin) by Perl's iron staining was observed on the surface of the spleen and liver 5 weeks after asbestos injection, which was aggravated by nitrilotriacetate (NTA) and ameliorated by desferal (bar = 50 μm). (b) Serum ferritin levels were in proportional association with iron deposition (means ± SEM). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

when necessary. Statistical analyses were carried out with Prism 5 (GraphPad Software Inc., San Diego, CA, USA).

Results

Protocol for animal experiments and body weight. To optimize the experimental protocol for rats, we carried out a preliminary 5-week experiment to determine the maximal non-toxic dose of desferal in order to avoid false negative results. Based on the results of the preliminary experiment, we injected rats with 100 mg/kg desferal i.p. once a week for a total of 5 weeks. We weighed the rats weekly and observed that repeated NTA treatment after asbestos injection caused body weight reduction in both the chrysotile and crocidolite

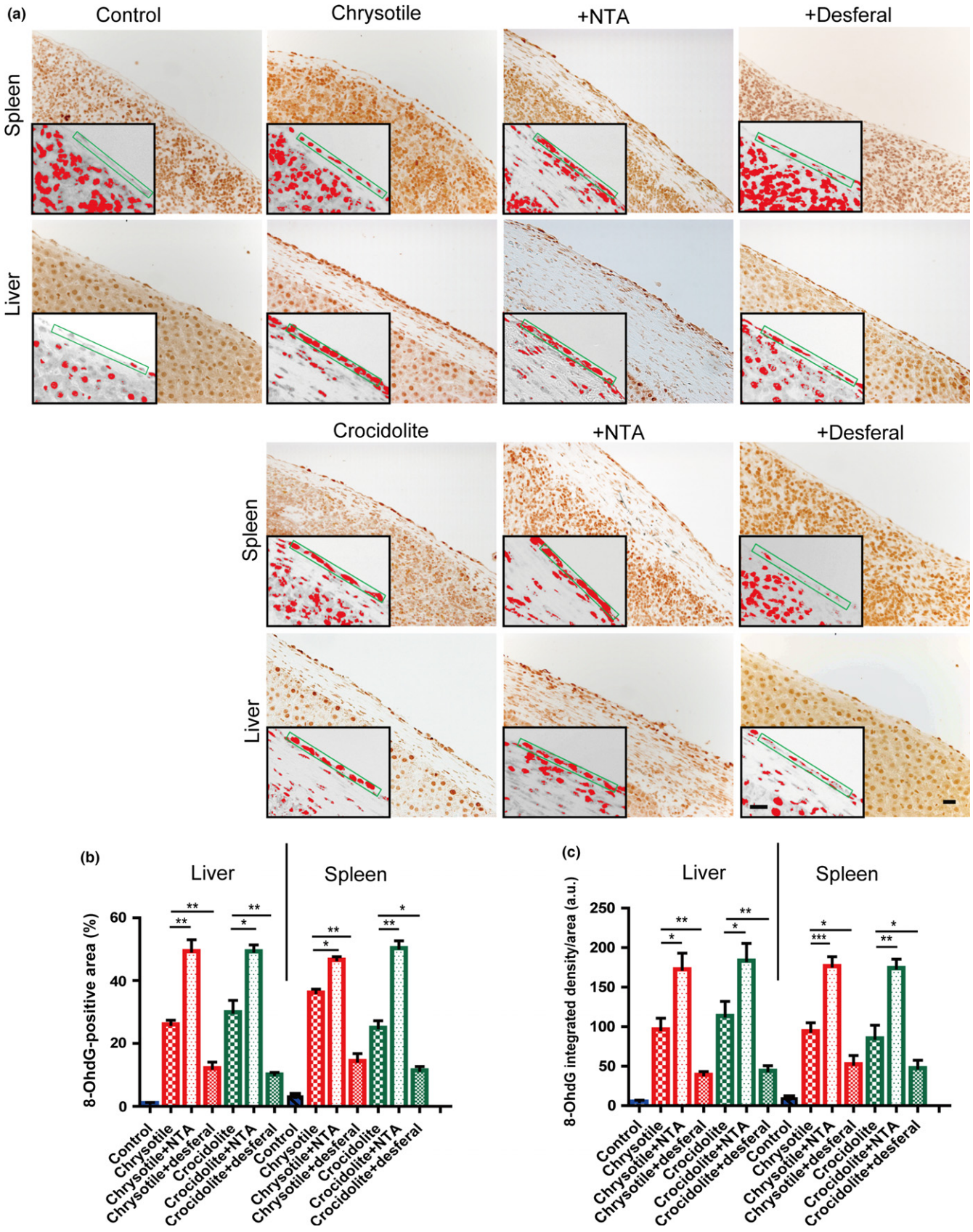


Fig. 4. Immunohistochemical analysis of 8-hydroxy-2′-deoxyguanosine (8-OHdG) in rat peritoneal mesothelial cells after asbestos injection in the presence or absence of iron chelators. (a) Nuclear immunostaining of surface mesothelial cells were observed 5 weeks after asbestos injection, which was aggravated by nitrilotriacetate (NTA) and ameliorated by desferal (bar = 50 μm). (b,c) Quantitative analysis of immunostained area (b) and integrated density per area (c) (means ± SEM). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. a.u., arbitrary unit.

groups (Fig. 1a), which could be attributed to the promotion of the Fenton reaction. In contrast, desferal treatment did not result in significant difference in body weight from the saline control group (Fig. 1a).

Desferal quenches asbestos-induced inflammation of somatic cavity. Asbestos injection with or without repeated NTA post-treatment constantly caused severe fibrous peritonitis, inducing dull edge and spherization of the lobes of liver (Fig. 1b). In contrast, post-treatment with desferal after asbestos injection counteracted these effects (Fig. 1b). Notably, rats treated with desferal after asbestos injection showed markedly reduced fibrosis on the serosal surface, whereas NTA enhanced it (Fig. 2a). For quantitation, we calculated the fibrosis index, which confirmed that the findings are statistically significant (Fig. 2b).

Desferal reduces asbestos-induced local iron deposition. Perl's iron staining on tissue specimens showed that desferal reduced stainable iron on the serosal surface of the liver and spleen, whereas NTA increased iron deposits (Fig. 3a). Asbestos injection caused an increase in the serum ferritin level compared to the control, which was further increased by NTA treatment, though desferal post-treatment reduced it (Fig. 3b).

Desferal inhibits asbestos-induced 8-OHdG generation in mesothelial cells. After i.p. injection of chrysotile or crocidolite, nuclear immunostaining of 8-OHdG was observed in the mesothelia covering the spleen and liver (Fig. 4a). Desferal post-treatment decreased immunostaining, whereas NTA increased it (Fig. 4a). Quantitation of 8-OHdG immunohistochemistry on mesothelial cells confirmed statistical significance among each group with the analysis of both area and integrated density (Fig. 4b,c).

Desferal decreases asbestos-induced cytotoxicity in mesothelial cells but not in macrophages. We evaluated cell viability after asbestos exposure by MTT assay in RPMC and RAW264.7. Desferal protected mesothelial cells from asbestos-induced cell injury, but this effect was not observed in macrophages (Fig. 5).

Desferal inhibits proliferation of MM, but not mesothelial cells. We carried out MTT assay *in vitro* using RPMC and two asbestos-induced rat MM cell lines, SM1 and SM2 derived from the sarcomatoid subtype,[25] for comparison. Desferal inhibited the growth of MM cells in a time- and dose-dependent manner (Fig. 6a). Two MM cell lines revealed similar results after 48-h treatment with 15–30 μM desferal. However, no similar effects were observed in RPMC after treatment with desferal.

In order to determine whether the inhibition of MM proliferation by desferal is iron-dependent, we treated the cells with 10 or 50 μg/mL holotransferrin with or without desferal (Fig. 6b). Cytostatic effects of desferal were partially ameliorated by the pretreatment with holotransferrin in SM cell lines. Pretreatment with holotransferrin alone showed no effects on cell growth of mesothelial or MM cells (Fig. 6b).

Discussion

In our previous chemoprevention experiment against asbestos-induced mesothelial carcinogenesis in rats, deferasirox (given orally) significantly induced mesenchymal epithelial transition in MM to a favorable histological subtype.[18] Thus, iron removal therapy was shown to be effective in asbestos-induced mesothelial carcinogenesis. However, due to potential side-effects of renal toxicity after repeated treatment, it is not ethically easy to apply this to humans who have been already exposed to asbestos.[26, 27] In the present study, we used another iron chelating agent, desferal, for this purpose.

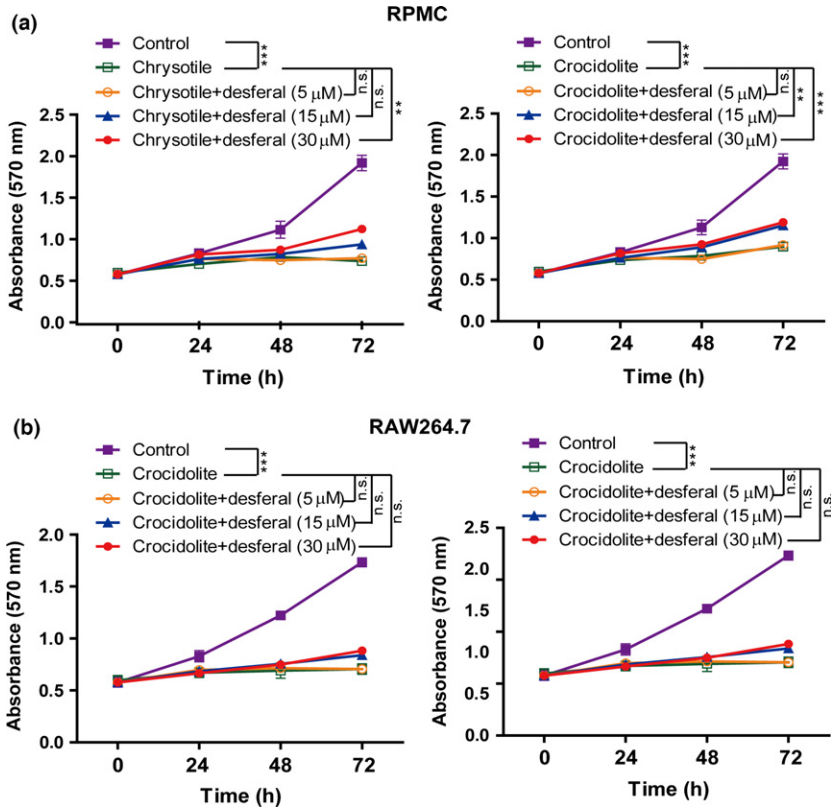


Fig. 5. Differential effect of desferal on asbestos-induced cytotoxicity between rat peritoneal mesothelial cells (RPMC) and murine macrophages (RAW264.7) by MTT assay. (a) Dose-dependent effects on mesothelial cells. (b) Effects on macrophage cells. Means ± SEM. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. n.s., statistically not significant.

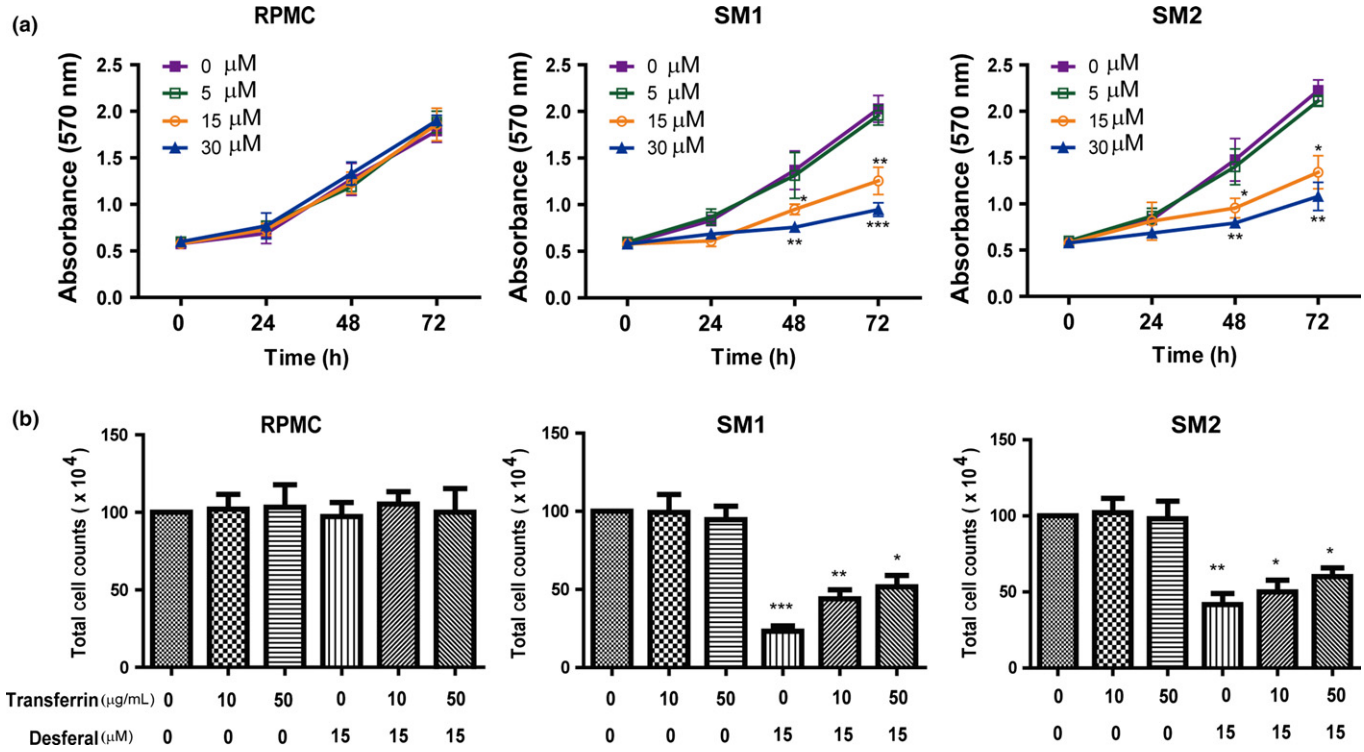


Fig. 6. Antiproliferative effects of desferal on malignant mesothelioma (MM) cells and its modulation by holotransferrin. (a) Desferal dose-dependently inhibited the proliferation of asbestos-induced rat MM cell lines (SM1 and SM2) but not rat peritoneal mesothelial cells (RPMC). (b) Pretreatment with holotransferrin partially reduced the inhibitory effects of desferal in MM cells. SM, sarcomatoid mesothelioma.

Desferal has been clinically used since the 1970s by the parenteral route for patients with iron overload, resulting from a variety of pathologies such as genetic hemochromatosis[28] and repeated transfusion.[29] We used peritoneal injections of asbestos to induce mesothelial carcinogenesis in the peritoneal cavity of rats.[6] In combination with *in vitro* experiments using cultured cells, our results here indicate dual merits of desferal against asbestos-induced mesothelial carcinogenesis, including inhibition of carcinogenesis and of the proliferation of established MM.

In our subacute experiments using rats, desferal significantly prevented asbestos-induced fibrosis of the peritoneal surface on the spleen and liver. Concomitantly, local iron deposition, serum ferritin representing general iron stores, and 8-OHdG levels of mesothelial cells were significantly reduced with desferal treatment. We also injected another iron chelating agent with an opposing effect, NTA, to enhance the iron-catalyzed Fenton reaction and compared the observed effects to those obtained with desferal. The results of NTA injection were opposite to those obtained with desferal, suggesting that iron deposition and inflammation are closely associated with fiber-induced carcinogenesis. Of note, our previous findings using various carbon nanotubes of different diameters indicated that the degree of fibrosis and iron deposits observed in subacute experiments is predictive of subsequent mesothelial carcinogenesis.[23,30]

Mechanistically, desferal was also effective against asbestos-induced toxicity in mesothelial cells *in vitro*, but not in macrophages. This suggests that the iron uptake and/or inhibition of iron release are key for asbestos-mediated cytotoxicity in mesothelial cells and that resistance to asbestos-induced cytotoxicity differs between mesothelial cells and macrophages, presumably due to the difference in iron metabolism.

Additionally, desferal inhibited the proliferation of MM cells. This was not observed in non-transformed mesothelial cells and was at least partially recovered by iron supplementation with holotransferrin. Accordingly, more iron is required for neoplastic growth than for non-neoplastic cells.[7] Indeed, desferal has been suggested as an anticancer agent in several cancers, including breast cancer.[31,32]

In conclusion, desferal presents anticarcinogenic potential against asbestos-induced carcinogenesis from two distinct aspects, anti-inflammation and antiproliferation. Unfortunately, there is a limitation in terms of application of this technique to humans due to the invasive nature of intrapleural administration. However, this technique may be applied intrapleurally at the diagnostic biopsy of pleura under thoracoscopy, or i.v. desferal may be used with other conventional chemotherapy or radiation. Further studies are warranted to determine the preventive *in vivo* activity of desferal against asbestos-induced mesothelial carcinogenesis.

Acknowledgments

This work was supported, in part, by the National Cancer Center Research and Development Fund (25-A-5), Grants-in-aid for Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (24390094; 221S0001-04; 24108008), and the Yasuda Medical Foundation. The authors wish to thank Nobuaki Misawa for excellent technical assistance with the pathologic specimens.

Disclosure Statement

The authors have no conflict of interest.

References

1 Roggli VL, Oury TD, Sporn TA (eds.). *Pathology of asbestos-associated diseases. 2 edn*. New York: Springer Verlag, 2004.
2 IARC, WHO. Asbestos (chrysotile, amosite, crocidolite, tremolite, actinolite, and anthophyllite). IARC Monographs on the Evaluation of Carcinogenic Risks to Humans A Review of Human Carcinogens; Part C: Arsenic, Metals, Fibres, and Dusts. Lyon, France, 2012; 219–309.
3 Robinson B, Lake R. Advances in malignant mesothelioma. *N Engl J Med* 2005; **353**: 1591–603.
4 Robinson B, Musk A, Lake R. Malignant mesothelioma. *Lancet* 2005; **366**: 397–408.
5 Thompson JK, Westbom CM, Shukla A. Malignant mesothelioma: development to therapy. *J Cell Biochem* 2014; **115**: 1–7.
6 Jiang L, Akatsuka S, Nagai H *et al.* Iron overload signature in chrysotile-induced malignant mesothelioma. *J Pathol* 2012; **228**: 366–77.
7 Toyokuni S. Iron-induced carcinogenesis: the role of redox regulation. *Free Radic Biol Med* 1996; **20**: 553–66.
8 Toyokuni S. Oxidative stress and cancer: the role of redox regulation. *Biotherapy* 1998; **11**: 147–54.
9 Toyokuni S. Iron and carcinogenesis: from Fenton reaction to target genes. *Redox Rep* 2002; **7**: 189–97.
10 Toyokuni S. Role of iron in carcinogenesis: cancer as a ferrotoxic disease. *Cancer Sci* 2009; **100**: 9–16.
11 Anderson RL, Bishop WE, Campbell RL. A review of the environmental and mammalian toxicology of nitrilotriacetic acid. *Crit Rev Toxicol* 1985; **15**: 1–102.
12 Toyokuni S, Sagripanti JL. Iron-mediated DNA damage: sensitive detection of DNA strand breakage catalyzed by iron. *J Inorg Biochem* 1992; **47**: 241–8.
13 Toyokuni S. Mechanisms of asbestos-induced carcinogenesis. *Nagoya J Med Sci* 2009; **71**: 1–10.
14 Toyokuni S. Iron overload as a major targetable pathogenesis of asbestos-induced mesothelial carcinogenesis. *Redox Rep* 2013; **19**: 1–7.
15 Nagai H, Ishihara T, Lee WH *et al.* Asbestos surface provides a niche for oxidative modification. *Cancer Sci* 2011; **102**: 2118–25.
16 Kubo Y, Takenaka H, Nagai H, Toyokuni S. Distinct affinity of nuclear proteins to the surface of chrysotile and crocidolite. *J Clin Biochem Nutr* 2012; **51**: 221–6.
17 Hu Q, Akatsuka S, Yamashita Y *et al.* Homozygous deletion of *CDKN2A/2B* is a hallmark of iron-induced high-grade rat mesothelioma. *Lab Invest* 2010; **90**: 360–73.
18 Nagai H, Okazaki Y, Chew SH, Misawa N, Yasui H, Toyokuni S. Deferasirox induces mesenchymal-epithelial transition in crocidolite-induced mesothelial carcinogenesis in rats. *Cancer Prev Res* 2013; **6**: 1222–30.
19 Anderson C, Danylchuk KD. The effect of chronic administration of trisodium nitrilotriacetate (Na3NTA) on the haversian remodelling system in dogs. *J Environ Pathol Toxicol* 1979; **3**: 413–20.
20 Toyokuni S, Sagripanti J-L. DNA single- and double-strand breaks produced by ferric nitrilotriacetate in relation to renal tubular carcinogenesis. *Carcinogenesis* 1993; **14**: 223–7.
21 Okada S, Hamazaki S, Toyokuni S, Midorikawa O. Induction of mesothelioma by intraperitoneal injections of ferric saccharate in male Wistar rats. *Br J Cancer* 1989; **60**: 708–11.
22 Toyokuni S, Tanaka T, Hattori Y *et al.* Quantitative immunohistochemical determination of 8-hydroxy-2′-deoxyguanosine by a monoclonal antibody N45.1: its application to ferric nitrilotriacetate-induced renal carcinogenesis model. *Lab Invest* 1997; **76**: 365–74.
23 Nagai H, Okazaki Y, Chew S *et al.* Diameter of multi-walled carbon nanotubes is a critical factor in mesothelial injury and subsequent carcinogenesis. *Proc Natl Acad Sci U S A* 2011; **108**: E1330–8.
24 Usami N, Fukui T, Kondo M *et al.* Establishment and characterization of four malignant pleural mesothelioma cell lines from Japanese patients. *Cancer Sci* 2006; **97**: 387–94.
25 Jiang L, Yamashita Y, Chew SH *et al.* Connective tissue growth factor and β-catenin constitute an autocrine loop for activation in rat sarcomatoid mesothelioma. *J Pathol* 2014; **233**: 402–14.
26 Kontoghiorghes GJ. Deferasirox: uncertain future following renal failure fatalities, agranulocytosis and other toxicities. *Expert Opin Drug Saf* 2007; **6**: 235–9.
27 Dubourg L, Laurain C, Ranchin B *et al.* Deferasirox-induced renal impairment in children: an increasing concern for pediatricians. *Pediatr Nephrol* 2012; **27**: 2115–22.
28 Tavill AS. Diagnosis and management of hemochromatosis. *Hepatology* 2001; **33**: 1321–8.
29 Propper RD, Cooper B, Rufo RR *et al.* Continuous subcutaneous administration of deferoxamine in patients with iron overload. *N Engl J Med* 1977; **297**: 418–23.
30 Nagai H, Okazaki Y, Chew SH *et al.* Intraperitoneal administration of tangled multiwalled carbon nanotubes of 15 nm in diameter does not induce mesothelial carcinogenesis in rats. *Pathol Int* 2013; **63**: 457–62.
31 Hoke EM, Maylock CA, Shacter E. Desferal inhibits breast tumor growth and does not interfere with the tumoricidal activity of doxorubicin. *Free Radical Biol Med* 2005; **39**: 403–11.
32 Rao VA. Iron chelators with topoisomerase-inhibitory activity and their anticancer applications. *Antiox Redox Signal* 2013; **18**: 930–55.