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Original Articles

Acute cytotoxicity of MIRA-1/NSC19630, a mutant p53-reactivating small molecule, against human normal and cancer cells via a caspase-9-dependent apoptosis

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ABSTRACT

Although numerous studies have focused on the mechanisms of action of the candidate chemotherapeutic drug MIRA-1/NSC19630, initially described as a mutant p53-reactivating small molecule, the issue of its toxicological evaluation remains open. Here, we devised a strategy to examine the effects of MIRA-1 on a variety of human normal cells and cancer cell lines. First, we demonstrated a massive and rapid (within 2 hours) MIRA-1 apoptotic effect on human normal primary epithelial cells as shown using an intestinal mucosa explant assay. MIRA-1 was also cytotoxic to primary and subcultured human mesenchymal cells. Interestingly these effects were restricted to actively proliferating cells. Second, MIRA-1 acute toxicity was independent of p53, since it occurred in human normal cells with increased or silenced p53 expression level, in cancer cells derived from solid or liquid tumors, with either mutated or wt TP53, and in cancer cells devoid of p53. Third, combined pharmacological and genetic approaches showed that MIRA-1 acute cytotoxicity was mediated by a caspase-9-dependent apoptosis. In conclusion, our strategy unveils the limitations of the targeted action of a small molecule designed to reactivate mutant p53.

Introduction

The toxicological evaluation of chemotherapeutic agents is a challenging issue as their efficacy is based on their cytotoxic potential. In fact, most of the so-called conventional chemotherapeutic drugs target the DNA replication machinery or the mitotic apparatus. It is thus not surprising that there are severe limitations to their use based on their heavy cytotoxicity burden. The adverse effects of a number of cytotoxic drugs are largely related to their ability to kill rapidly proliferating cells such as bone marrow and/or gut cells. Chemotherapy causes apoptosis of intestinal crypts followed by crypt hypoplasia [1].

The discovery of molecular alterations specifically associated with a given cancer type and driving cancer progression has raised hopes of a new era of anti-cancer treatments based on targeted chemotherapy [2]. This is best illustrated by imatinib, a small molecule that selectively interferes with the BCR-Abl tyrosine kinase that is specifically found in chronic myelogenous leukemia and leads to the apoptotic death of leukemic cells without altering normal cells. In this regard, imatinib is one of the first cancer therapies to show the potential for such targeted action, and therefore exemplifies a paradigm of targeted therapies [3]. However even such a targeted therapy causes side effects including hematologic, cutaneous, digestive or neurologic toxicities which can lead to dose reduction or discontinuation of imatinib treatment [4]. This example illustrates that even targeted therapies can have significant side effects or adverse events [5].

The TP53 tumor suppressor encodes a transcription factor whose activation upon DNA damage or oncogenic stresses results in cell cycle arrest or senescence or apoptosis. TP53-inactivating mutations are among the most studied molecular alterations in cancer because they occur in nearly 50% of human tumors [6]. TP53 mutations not only cause loss of wild type properties but also endow

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the protein with so-called gain-of-function activities including inhibition of apoptosis [6]. In this context, finding a drug able to restore the normal function of p53 is a crucial challenge in cancer research, because such a drug could theoretically exert a selective pro-apoptotic effect on cancers bearing mutated TP53 without harmful effects on normal cells. Interestingly, MIRA-1/NSC19630 (1-propoxymethyl-maleimide) was first identified as a mutant p53-reactivating agent through the screening of the Diversity set of low molecular weight compounds from NCI, National Institutes of Health, using p53-null Saos-2 osteosarcoma cells carrying a tetracycline-regulated R273H mutant p53 (Tet-off) [7]. MIRA-1 induced apoptosis of Saos-2-R273H cells expressing mutant p53 but did not significantly affect growth of these cells cultured in the presence of doxycycline that shuts off mutant p53 expression. Using the same tet-off strategy combined with a WST-1 viability assay, this compound was also shown to induce at a 25 μM concentration a mutant p53-dependent growth inhibition in human SKOV ovarian carcinoma carrying His175 or His273 mutant p53 under the control of the tetracycline-dependent promoter [7]. In addition MIRA-1 could also restore transcriptional transactivation to mutant p53 in several human carcinoma cell lines [7]. In vivo efficacy was studied in xenotransplanted SCID mice after intraperitoneal injection of the structural MIRA-1 analog MIRA-3, which revealed potent antitumor effect and significantly reduced tumor growth. Toxic side effects including diarrhea were only observed at very high doses that largely exceeded the therapeutically effective dose of MIRA-3, suggesting that mutant TP53 is druggable by the p53-reactivating MIRA compounds [7], at least in cell types derived from solid malignant tumors.

Intriguingly, in a more recent study aimed at evaluating the effect of MIRA-1/NSC19630 on the NCI 60 cancer cell lines, using the WST-1 assay, an 80% growth inhibition of U2OS cells harboring a wild-type TP53 gene was observed upon a two day exposure to MIRA-1/NSC19630 at a concentration as low as 3 μM [8]. As to whether this inhibitory effect is cell line specific is not known. Increasing the complexity, the p53 mutation status was recently reported not to interfere with the antiproliferative and apoptotic effects of MIRA-1 in a series of liquid tumors, i.e. myeloma cell lines and primary cells from multiple myeloma patients [9]. However, in this paper, the TP53 mutation status of the cell lines was inferred from the existing literature and not verified in the experimental design. In addition, the TP53 status of primary fresh cells from the patients was unknown. Furthermore, in a 48 h in vitro viability assay, some MIRA-1 cytotoxicity was detected in normal hematopoietic cells. Together, the studies of Aggarwal et al. [8], and Saha et al. [9], raise the issue of a mutant p53 selective mechanism of action of MIRA-1 on several tumor cell types. In addition, the study by Saha et al. raises some doubts about the claimed absence of MIRA-1 cytotoxicity to normal cells. These reports prompted us to engage an in-depth investigation of the effects of MIRA-1 on human normal epithelial and mesenchymal cells, as well as on human tumor cell lines of several origins with a verified TP53 status.

Here we designed an approach that can accurately and reproducibly mimic at its best the in vivo situation in human. To this end, an ex vivo culture system of human intestinal mucosa was recently developed that maintains the 3D dimensional structure of the human intestinal crypt [10,11]. In this model the epithelial cells maintain the spatio-temporal hierarchy of proliferation and differentiation, with the actively dividing cells restricted to the lower part of the crypt and the post-replicative cells present in the upper part. When used in combination with an immunohistochemical marker of epithelial apoptosis (M30 antibody) [12,13] or with the TUNEL assay, this culture system is a valuable tool to evaluate the acute apoptotic effects of a given stress on proliferating and non-proliferating human normal cells in the same assay [10]. Using this approach, we demonstrate here that MIRA-1, at concentrations previously reported to induce apoptosis selectively in TP53 mutant cells, is able to induce a rapid (within 2 hours) and massive apoptosis in proliferating human normal epithelial cells. This effect was then extended to normal cells of mesenchymal origin and was found independent from the p53 expression level. In addition, we examined in depth the role of TP53 mutation status on the acute MIRA-1 cytotoxicity in a series of human cancer cell lines representing solid and liquid tumors, whose TP53 mutation status was verified. Using a combination of pharmacological and genetic approaches based on a set of cell lines, we provide experimental evidence that the MIRA-1 acute cytotoxic effect is independent of the TP53 mutation status. Finally, it results from activation of the apoptosome–caspase-9 pathway of apoptosis.

Materials and methods

Human colonic mucosa explant cultures

Human normal colon was obtained from 5 patients undergoing surgery for colon carcinoma (4 men and 1 woman; 58–77 years; 3 proximal colon; 2 distal colon). The tissue fragments were processed according to the French guidelines for research on human tissues. Informed patient consent was obtained according to the French bioethics law. The mucosa was carefully stripped from the underlying compartment, and fragments (40 mg wet weight) were maintained in 2 ml culture medium (RPMI/BSA/antibiotics–fungizone) [10] for 3 hours with or without MIRA-1 (12.5 or 25 μM, Tocris). Some explants were concomitantly treated with MIRA-1 and the pan-caspase inhibitor zVAD (10 μM, BD Pharmingen). The explants were maintained at 37 °C in a 95% oxygen, 5% carbon dioxide humid atmosphere on a rocking platform at low speed. In each experiment, at least 3 explants were cultured per condition. After 3 hours, the mucosa explants were fixed in 10% buffered formalin and embedded in paraffin. Paraffin sections were subjected to standard hematoxylin–eosin (HE) staining and to immunohistochemistry using Bond Max automated immunohistochemistry staining system (Leica), according to the manufacturer's protocol. After antigen retrieval in citrate buffer, the M30 cytodeath antibody was applied (1:200, Roche Diagnostics). M30 antibody recognizes a cytokeratin-18 neoepitope that becomes available at an early caspase cleavage event during apoptosis and is considered an early marker of apoptosis in epithelial cells [12,13]. The percentage of colonic crypts containing at least 30% M30-positive cells in the lower third of the crypt was assessed by counting a total of about 100 well-oriented crypts at a 200× magnification. In addition, apoptosis was assessed by the TUNEL assay, which detects apoptotic cells by labeling DNA strand breaks, according to the manufacturer's protocol (Apoptag Peroxidase In Situ Apoptosis Detection Kit; Chemicon).

Primary cell culture and cancer cell lines

Primary human normal fibroblasts were isolated from the dermis of juvenile foreskin after collagenase digestion (0.1% in RPMI, 1 hour at 37 °C), and then maintained in culture in Dulbecco's Modified Eagle's Medium (DMEM), 4.5 g/l glucose/10% heat-inactivated FCS (InVitrogen, France). They were used between passages 1 and 10.

Several human cancer cell lines of different tissue origin, representative of different TP53 status as mentioned on the TP53 website (http://p53.free.fr), were used in this study: colorectal cancer cell lines (HCT116, SW48, Caco-2), and Jurkat, a T-cell leukemia cell line. In addition, we used a clonal derivative of the colonic HT29 cell line (HT29-Cl.27H) [14], a gastric cell line (HGT-1.01) [15], as well as 2 clones derived from Jurkat cell line (the caspase-9 deficient and proficient clones JMR and F9, respectively) [16]. Cell lines were maintained in standard culture medium (DMEM/10% heat inactivated FCS), with the exception of Jurkat cell line E6.1 and its clonal derivatives that were maintained in RPMI/10% heat inactivated FCS. All cultures were always found to be free of mycoplasma, as assessed by the method of Chen [17]. We verified and/or assessed the TP53 status for all these cell lines (see below).

Assessment of TP53 status

The TP53 status of the cell lines was verified by conventional Sanger sequencing. Five fragments spanning exons 5–9 and coding the DNA-binding domain were amplified by PCR, purified, and bidirectionally sequenced, using the same home-made primers (sequences available on demand) and the Big Dye Terminator kit on the Applied Biosystems 3130xl Genetic Analyser (Applied Biosystems, Foster City, CA, USA). Data were analyzed by visual inspection of electropherograms on Seqscanner software and compared to the reference sequence NG_017013 (NCBI Nucleotide) using Seqscape software (Applied Biosystems, Foster City, CA, USA). The variant allele frequency was determined manually at each point mutation by calculating the ratio of the peaks heights of the mutated allele relative to the sum of the mutated allele and the wild-type allele. The detection threshold of mutant allelic frequency was 10–15%. The functional analysis of each identified TP53 mutations was performed using the IARC database R16 release, November 2012 (http://www-p53.iarc.fr/) [18]. The characteristics of the cell lines used as well as their TP53 status are mentioned in Table 1.