Pharmacological inhibition of macrophage migration inhibitory factor interferes with the proliferation and invasiveness of squamous carcinoma cells

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Abstract. Recent clinical observations and experimental studies of our group indicate that macrophage migration inhibitory factor (MIF) may contribute to tumor progression in head and neck squamous cell carcinomas (HNSCC). The present study was undertaken to examine the effects of the irreversible MIF inhibitor 4-iodo-6-phenylpyrimidine (4-IPP) on proliferation and invasiveness of the squamous carcinoma cell line SCCVII. Cell counting, crystal violet assay and flow cytometry were used to analyze the effects of 4-IPP on SCCVII cell growth. The impact of 4-IPP on cell invasiveness was assessed by Boyden chamber assay. Knockdown of the MIF receptor CD74 was achieved by transduction with lentiviral vectors encoding anti-CD74 shRNAs. As shown by immunofluorescence staining, SCCVII cells express both MIF and CD74. Decreased MIF immunoreactivity as a result of exposure to 4-IPP suggested a covalent modification of the cytokine. 4-IPP inhibited SCCVII cell proliferation and invasiveness. Moreover, the cytostatic effect of 4-IPP was enhanced by CD74 knockdown. The inhibitory effects of 4-IPP on cell proliferation and invasiveness strongly suggest that MIF is involved in proliferative activity and invasive properties of squamous carcinoma cells. In conclusion, MIF inhibition may open possibilities for target-directed treatment of head and neck squamous cell carcinoma.

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Introduction

Macrophage migration inhibitory factor (MIF), originally named based on its repressive action on the motility of monocytes/macrophages, belongs to the cytokine family of signaling molecules. Although its existence as an extracellular regulator had been postulated since the late 1950s, it was not until 1989 that MIF was formally identified thanks to cDNA cloning and sequencing (1). A few years later the three dimensional structure of MIF was elucidated by structural analysis of the pure recombinant protein (2-4).

MIF is primarily known as a pro-inflammatory cytokine released by several cell types of the immune system, in particular macrophages and T cells, although its production is by no way restricted to immune cells. In addition to its paracrine activity, it can also act in endocrine fashion when secreted by corticotrophic cells of the pituitary (5). Physiologically, MIF has been shown to counteract the anti-inflammatory activivity of glucocorticoids at various levels, thus keeping a balance between pro-inflammatory and anti-inflammatory mechanisms (6). Expectedly, a dysregulation/exacerbation of MIF activity has been implicated in pathological conditions as diverse as asthma (7), chronic colitis (8), rheumatoid arthritis (9), systemic lupus erythematosus (10), psoriasis (11) and non-insulin-dependent diabetes mellitus (12).

As also seen for other cytokines such as interleukin-6, evidence is growing that MIF, beside its role in immune responses, could also be involved in neoplastic transformation and tumor progression. Among the earliest experimental observations that MIF might contribute to tumor development was the negative impact of MIF immunoneutralization on neoplastic growth in a mouse model of B cell lymphoma (13).

In the context of human cancer, MIF increase associated with neoplastic diseases was first suggested by ligand histochemistry using sarcolectin (14-16), soon followed by histochemical demonstration of MIF overexpression in prostatic adenocarcinoma metastases (17). More recent studies have reported MIF overexpression in a variety of human

cancers, such as gastric adenocarcinoma (18), ovarian cancer (19), colorectal carcinoma (20) and cervical cancer (21). In the case of head and neck squamous cell carcinomas (HNSCC), studies by our group have disclosed an enhanced MIF expression in oral cavity, laryngeal and hypopharyngeal squamous cell tumors (22-24). Of particular interest, stable MIF knockdown in a murine ovarian cancer cell line has been shown to decrease tumor burden and confer longer survival in tumor transplanted mice (19). A similar result was obtained after administration of anti-MIF therapeutics, either MIF-neutralizing antibodies or the MIF inhibitor ISO-1, to mice grafted with colorectal carcinoma transplants (20).

MIF exhibits a non-physiologic dopachrome tautomerase activity which has been extensively exploited to screen for potential inhibitors, owing to the fact that compounds antagonizing this enzymatic activity are also likely to disrupt MIF interactions with cognate receptors such as CD74 and suppress thereby its effects on target cells (25). Along this line, a new potent inhibitor of MIF dopachrome tautomerase activity, 4-iodo-6-phenylpyrimidine (4-IPP) has been shown to decrease the motility and anchorage-independent growth of lung adenocarcinoma cells (26).

Since MIF has been reported to be overexpressed in head and neck squamous cell carcinomas (22-24), the present study was undertaken to examine possible inhibitory effects of 4-IPP on SCCVII squamous carcinoma cells.

Materials and methods

Inhibitor and antibodies. MIF inhibitor 4-iodo-6-phenylpyrimidine (4-IPP) was purchased from Tocris Bioscience (Bristol, UK). Stock solutions of the compound, prepared ≥250-fold concentrated in ethanol, were stored at -20˚C and used within the month. Polyclonal antibody against purified MIF (14) was raised in rabbits. Serum IgG fraction was purified as described previously (24). Rabbit polyclonal anti-CD74 antibody (FL-296), raised against residues 17-296 of human CD74, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cell culture medium and supplements were from Lonza (Verviers, Belgium). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Other chemicals were from standard commercial sources.

Cell line and culture. The squamous cell carcinoma cell line SCCVII (27) was a kind gift from Dr Shulin Li (Louisiana State University). Routine cell propagation and experimental studies were carried out at 37˚C in a cell incubator with humid atmosphere at 5% CO_2. Unless specified otherwise, cells were cultured in T-flasks containing Dulbecco's modified essential medium (DMEM) supplemented with phenol red, 10% FBS, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 2 mM L-glutamine and 1X antibiotic/antimycotic mix.

A CD74 knockdown (CD74-KD) cell line derived from SCCVII, as well as a matched control with normal CD74 expression, were generated in our laboratory. Knockdown of CD74 expression was achieved by using anti-CD74 shRNA (mouse) lentiviral particles from Santa Cruz. Controls were obtained by transduction with shRNA lentiviral particles encoding a scrambled (sc) shRNA sequence (CD74sc). Transduced cells were selected by addition of 4 μg/ml puromycin to the culture medium. Preliminary experiments showed this puromycin concentration to be toxic toward non-transduced SCCVII cells.

Western blotting. Transduced SCCVII cells (CD74sc and CD74KD) were plated in Petri dishes (10^6 cells/dish), cultured for 3 days and then lysed using a detergent cocktail (M-PER Mammalian Extraction buffer) supplemented with protease (Halt Protease Inhibitor Cocktail) and phosphatase inhibitors (Halt Phosphatase Inhibitor Cocktail) (all from Pierce, Rockford, IL, USA). Protein concentrations were determined by the BCA Protein assay (Pierce) using bovine serum albumin as standard. Extracted proteins (30 μg) were subjected to 10% SDS-PAGE and electrotransferred onto nitrocellulose membranes (iBlot® Dry Blotting system, Life Technologies-Invitrogen, Gent, Belgium). Immunodetections were performed using anti-CD74 antibody FL-296 (1/200). Peroxidase-labeled anti-rabbit IgG antibody (1/5,000) (from Amersham Pharmacia Biotech, Roosendaal, The Netherlands) was used as secondary reagent. Bound peroxidase activity was revealed using the SuperSignal® West Pico Chemiluminescent Substrate (Pierce) following the manufacturer's instructions. Immunostaining signals were digitalized with a PC-driven LAS-3000 CCD camera (Fujifilm, Tokyo, Japan), using a software specifically designed for image acquisition (Image Reader, Raytest®, Straubenhardt, Germany). Immunoreactive band intensities were quantified using the software AIDA® Image Analyser 3.45 (Raytest).

Measurement of cell culture growth by cell counting. SCCVII cells were plated at a density of 10^4 cells/cm^2 in 12-well dishes. The next day, cell cultures were fed fresh medium with or without 4-IPP (concentrations specified in Results). Measurement of cell culture density was performed either daily (time-course experiment) or after 3 days of treatment. Cells were dislodged from the vessel bottom by treatment with trypsin-EDTA solution (Lonza). After vigorous pipetting, concentrations of cells in suspension were determined in an electronic cell counter (model Z1 Coulter counter, Beckman Coulter, Fullerton CA).

Measurement of cell culture growth by crystal violet stain assay. Cell density was also assessed by colorimetry after crystal violet staining, as described previously (28). Cells were seeded in 96-well plates (1,000-2,000 cells/well). The following day, they were fed fresh medium (DMEM, 10% FBS) with or without 4-IPP. After a 3-day exposure, the culture medium was discarded and cells were fixed with 1% glutaraldehyde. Following fixation, cells were stained with 1% crystal violet. Destaining was achieved under gently running tap water and cell monolayers were lysed in 0.2% Triton X-100. The absorbance of cell lysates was measured at 570 nm using a Labsystems Multiskan MS microplate reader.

Measurement of cell culture growth by bromodeoxyuridine incorporation. DNA synthesis in SCCVII cells was evaluated on the basis of bromodeoxyuridine (BrdU) incorporation measured using an enzyme immunoassay kit developed by Roche Diagnostics (Mannheim, Germany). The assay was performed following the manufacturer's instructions, with minor modifications. Briefly, SCCVII cells were plated in 96-well plates (2,000 cells/well) and treated the following

day with 4-IPP. For the assessment of BrdU incorporation into DNA, the cell cultures were exposed for 50 min to BrdU labelling solution, followed by fixation and denaturation with an ethanol-based mixture. Thereafter cells were incubated for 30 min in a blocking solution (Animal-Free Blocker, Vector Laboratories, Burlingame, CA). The next step consisted in incubating cells in the presence of an anti-BrdU antibody conjugated with horseradish peroxidase. Immunocomplexes were detected by using a mixture of H_2O_2/tetramethylbenzidine as substrate. After addition of the stop solution (1 M H_2SO_4), the absorbance of the samples was measured at 450 nm (reference wavelength, 690 nm) in a microplate reader.

Cell cycle analysis by flow cytometry. The effect of 4-IPP on SCCVII cell cycle was examined using the Cell Cycle Phase Determination kit from Cayman Chemical Co. (Ann Arbor, MI). Cells were seeded in 6-well plates (10,000 cells/well) and cultured for 24 h prior to exposure to 10, 20 or 40 μM 4-IPP or vehicle for 48 h. After treatment, cells were collected (trypsin-EDTA solution), centrifuged (500 x g, 5 min) and washed twice with assay buffer (Cayman Chemical Co.) before incubation in fixative solution (Cayman Chemical Co.) for at least 2 h. Fixed cells were centrifuged, suspended in 0.5 ml staining solution (propidium iodide and RNase solution) (Cayman Chemical Co.) and incubated for 30 min at room temperature in the dark. Finally, the samples were analyzed in the FL2 channel of a flow cytometer (FACSCalibur, Becton-Dickinson, Franklin Lakes, NJ) equipped with a 488-nm excitation laser.

Evaluation of MIF immunoreactivity by enzyme-linked immunosorbent assay. The immunoreactivity of MIF protein was assessed by a sandwich enzyme-linked immunosorbent assay (ELISA) using a commercial kit (DuoSet ELISA Development kit, R&D Systems, Minneapolis, MN). Ninety-six well ELISA plates were coated overnight with 100 μl/well of capture antibody (mouse anti-human MIF). After 3 rinses of the wells with washing buffer (PBS, 0.05% Tween-20), non-specific binding was prevented by adding the reagent diluent containing 1% BSA and incubating for 1 h. The above mentioned rinsing procedure was applied between all subsequent steps. The blocking step was followed by the addition of increasing concentrations of human recombinant MIF in reagent diluent. After an incubation period of 2 h, 100 μl of detection antibody (biotinylated goat anti-human MIF) were added to each well. A 2-h exposure to the detection antibody was followed by a 20-min incubation with 100 μl of horseradish peroxidase-conjugated streptavidin. Immunocomplexes were revealed by the addition of 100 μl substrate solution (H_2O_2/tetramethylbenzidine). After stopping the reaction with H_2SO_4, the absorbance of the samples was measured at 450 nm (reference wavelength, 570 nm) as described above.

Immunofluorescence microscopy. SCCVII cells were plated at a density of 5,000 cells/cm^2 on sterile round glass coverslips in 12-well dishes. The following day, they were fed fresh medium with or without 20 μM 4-IPP. After 3 days of drug exposure, cell monolayers were fixed with 4% paraformaldehyde in Dulbecco's PBS (DPBS). Following fixation, paraformaldehyde was changed for DPBS where cell cultures were stored at 4˚C until immunostaining. Before application of antibodies, cell monolayers were rinsed several times with PBS (0.04 M Na_2HPO_4, 0.01 M KH_2PO_4, 0.12 M NaCl, pH 7.2) containing 0.1% Triton X-100 (the same detergent-containing buffer was used for subsequent incubations and rinsing steps). Prior to exposure to primary antibodies, cells were preincubated for 20 min in PBS containing 0.05% casein and 0.05 M NH_4Cl to prevent non-specific adsorption of immunoglobulins. Cells were exposed for 60 min to the primary antibody (either anti-MIF or anti-CD74) diluted 1:50 in PBS containing 0.05% casein. This was followed by a 30-min exposure to biotinylated goat anti-rabbit IgG (Vector Laboratories). Thereafter, the cell preparations were incubated for 30 min in the presence of Texas Red-conjugated streptavidin (Vector Laboratories). After final rinses in PBS, the coverslips were mounted on glass slides using commercial anti-fading medium (Vectashield®, Vector Laboratories). Negative controls were produced by omitting the incubation step with primary antibodies. This modification resulted in a decrease of the fluorescence signal to background level.

The cell preparations were examined on a Leitz Orthoplan microscope equipped with a Ploem system for epi-illumination. Excitation wavelength of 560 nm and emission wavelength of 590 nm were used for the observation of Texas Red fluorescence. Image capture was achieved using a PC-driven digital camera (Leica DC 300F, Leica Microsystems AG, Heerbrugg, Switzerland) operated with Leica IM50 software. Quantitative analysis of fluorescence signals was performed on digitalized images thanks to Image J™ (a public domain image software developed by W. Rasband at the Research Services Branch of the National Institute of Mental Health, NIH). Images were analyzed in the red channel after RGB split. Gray levels (on a scale of 0-225), corresponding to fluorescence intensity were determined in 65 cells in each control or treated cultures. Distribution histograms were plotted using Sigma Plot (Systat Software, Inc., Hounslow, UK). Confocal microscopy observations were carried out using an Olympus FV1000D laser scanning inverted microscope equipped with a red laser diode (LD559).

Phase contrast microscopy. Appearance of live cells in cultures was documented by phase-contrast microscopy using a Wilovert inverted microscope (Leitz, Wetzlar, Germany) equipped with a Leica DC 300F digital camera (see above).

Cell migration assay. Cell invasiveness was assessed by a Boyden chamber assay consisting of 24-well plates (lower chambers) with cell culture inserts (upper chambers), both chambers being separated by a polycarbonate membrane (8-μm size) coated with an artificial extracellular matrix (Chemicon Cell Invasion Assay kit, Millipore, Billerica, MA). The kit was used according to the manufacturer's instructions. SCCVII cells were seeded in cell culture inserts ($1.5x10^5$ cells/insert) in serum-free medium (DMEM). The lower chambers were filled with complete medium (DMEM, 10% FBS) with or without 40 μM 4-IPP (n=4). After 96 h, cells were wiped off the upper surface of cell culture inserts with a cotton-tipped swab and the migrating cells were stained with crystal violet and counted in 6 microscopic fields (magnification x10) using a Zeiss Axio scope A1 microscope (Carl Zeiss, Germany).

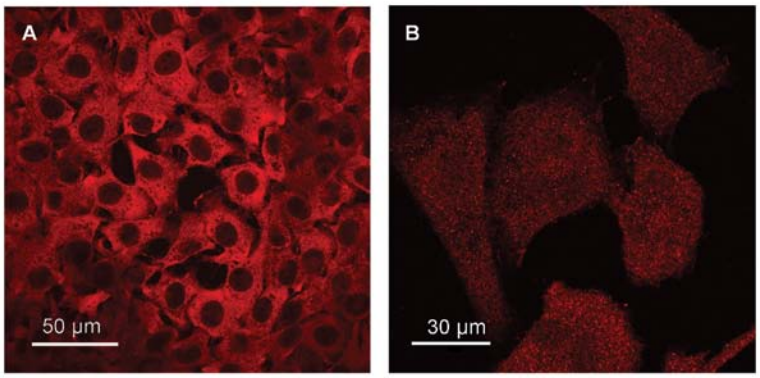


Figure 1. Demonstration of MIF (A) and CD74 (B) in SCCVII cells by immunofluorescence microscopy. Texas Red labelling, confocal microscopy.

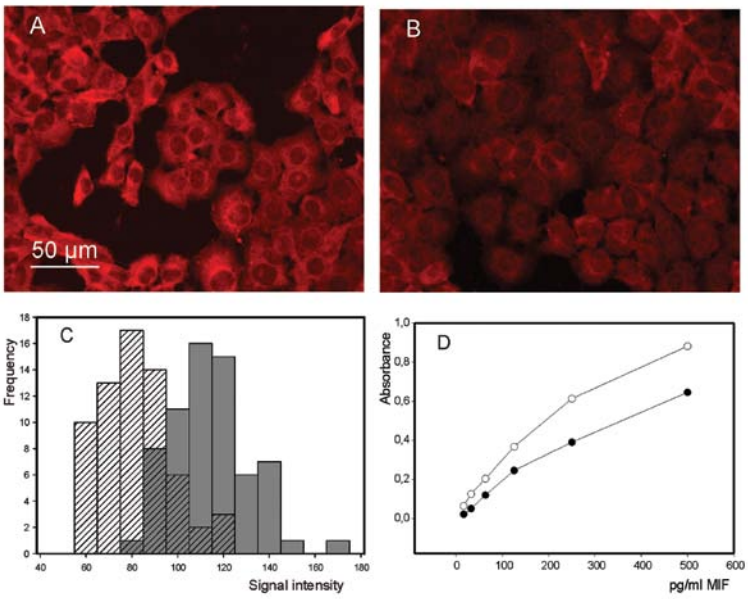


Figure 2. Alteration of MIF immunoreactivity by 4-IPP. (A and B) Intensity of MIF immunofluorescence signal in untreated cells (A) and cells treated for 3 days with 4-IPP (20 μM) (B). (C) Distribution histogram of fluorescence intensities for untreated cells (grey bars) and treated cells (hatched bars). (D) MIF immunoreactivity assessed by ELISA (duplicate measurements) after a 24-h exposure to 4-IPP (40 μM) (filled symbols). Open symbols, human recombinant MIF assayed in parallel in absence of 4-IPP.

Statistical analyses. SigmaPlot® 11 software was used for statistical analyses. Parametric analysis was performed by ANOVA (>2 groups, pairwise comparisons achieved by the Holm-Sidak method) or Student's t-test (2 groups). Non-parametric analysis used the Mann-Whitney rank sum test.

Results

SCCVII cells express both MIF and CD74. Demonstration of MIF in SCCVII cells by immunofluorescence microscopy is illustrated in Fig. 1A. MIF immunoreactivity is extranuclear and appears distributed throughout the cytoplasm of cells. Unlike that recently reported for MIF distribution in glioblastoma cells (29), the current data give no evidence of a prominent perinuclear localization. Similar to MIF, CD74 seems to be expressed by most, if not all SCCVII cells. Immunoreactive CD74 exhibits a punctuated pattern on the whole cell surface (Fig. 1B), a distribution consistent with the membrane localization of the receptor.

4-IPP alters MIF immunoreactivity. Fluorescence microscopy examination of MIF in 4-IPP-treated cells revealed a decrease in the intensity of immunofluorescence signal (Fig. 2A and B), further confirmed by image analysis (Fig. 2C). Even though this observation is suggestive of a decrease in MIF expression, the inhibitory action of 4-IPP should not be expected to affect cell MIF content. Yet, it is well known that 4-IPP, like other irreversible MIF inhibitors, reacts covalently with the N-terminal proline of MIF (26), inducing thereby a conformational change of the protein that could modify its immunoreactivity. Thus, we checked MIF immunoreactivity by enzyme-linked immunoassay in presence and absence of 4-IPP. As revealed by data presented in Fig. 2D, the addition of 4-IPP resulted in a marked decrease of absorbance associated with the formation of immunocomplexes, clearly showing that 4-IPP drastically alters MIF immunoreactivity.

4-IPP inhibits the proliferation of SCCVII cells. Routine examination of SCCVII cell cultures exposed to 4-IPP

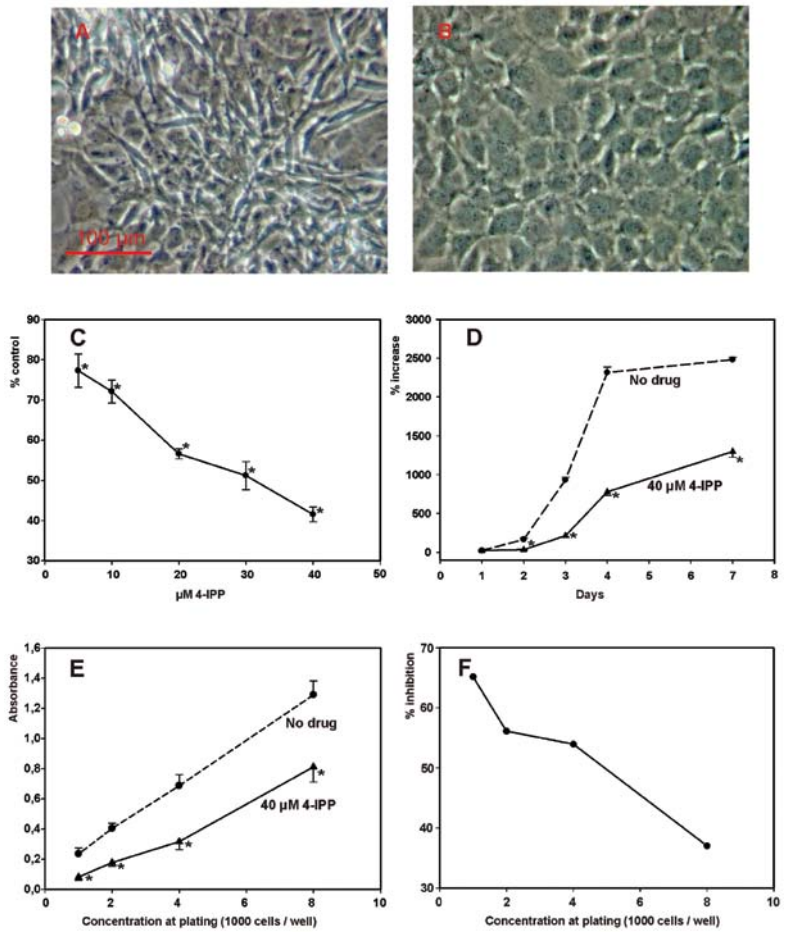
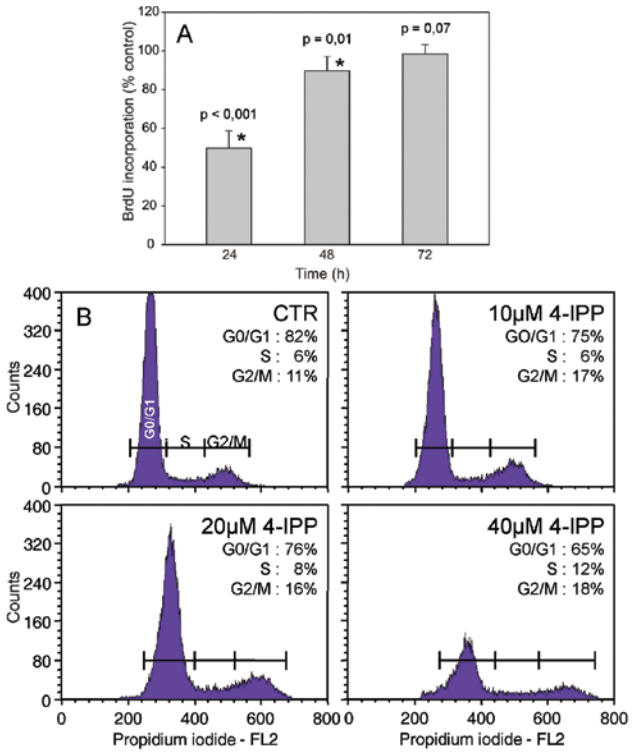


Figure 3. Effect of 4-IPP on SCCVII cell proliferation. (A and B) Appearance of cell cultures 7 days after plating. (A) No drug. (B) Cells grown in presence of 40 μM 4-IPP (6 days). (C) Dose-effect relationship (cells counted after a 3-day exposure to 4-IPP, each symbol is the mean of 4 determinations ± SD) (ANOVA, differences versus control assessed by Holm-Sidak method, $^{*}p<0.001$). (D) Time-course of cell culture growth (% increase in cell number determined by cell counting, each symbol is the mean of 4 determinations ± SD) (differences versus controls assessed by Student's t-test, $^{*}p<0.001$). (E and F) Inhibition of cell culture growth, as assayed by crystal violet staining. (E) Absorbance relative to cell concentration at plating (each symbol is the mean of 6 determinations ± SD) (differences versus controls assessed by Student's t-test, $^{*}p<0.001$). (F) Percent inhibition as a function of cell concentration at plating, calculated from data (E).



(Fig. 3A and B) suggested that the MIF inhibitor might exert an inhibitory effect on cell proliferation. Thus, cell culture growth studies were conducted in order to disclose a possible effect of MIF inhibition on cell proliferation. As illustrated in Fig. 3C, 4-IPP provoked a dose-dependent decrease of cell culture growth with an IC_{50} ~30 μM. At 40 μM 4-IPP, its inhibitory action on cell proliferation was already apparent after 1 day of exposure (Fig. 3D). Observations based on cell counting were confirmed by crystal violet stain assay (Fig. 3E). In the latter assay, the extent of the cytostatic effect produced by 4-IPP was inversely related to cell plating concentration (Fig. 3F). IPP-4-induced decline in cell proliferation prompted us to examine the effect of the MIF inhibitor on DNA synthesis and the cell division cycle. Cell treatment with 4-IPP resulted in a transient reduction of BrdU incorporation into DNA, which lasted 2 days after drug addition (Fig. 4A). As shown by flow

Figure 4. Effect of 4-IPP on DNA synthesis and the cell cycle. (A) Inhibition of BrdU incorporation into DNA by 4-IPP (40 μM) as function of the duration of drug exposure (each symbol is the mean of 6 determinations ± SD) (data analysis by Student's t-test). (B) Impact of 4-IPP on SCCVII cell cycle. Flow cytometry analysis performed after 2 days of drug exposure.

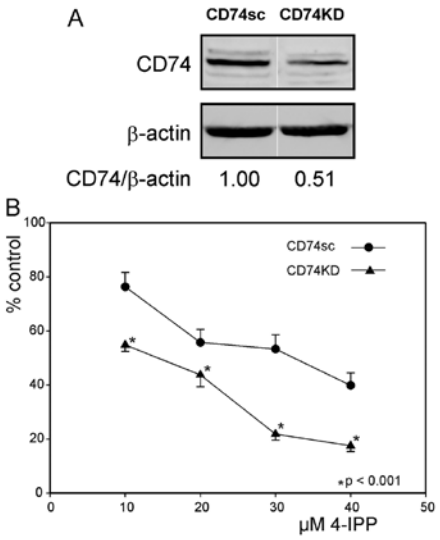


Figure 5. (A) Demonstration by western blotting of CD74 reduction obtained by shRNA transduction in SCCVII cells. (B) Dose-dependent inhibition of cell culture growth in SCCVII with CD74 knockdown (CD74KD) as compared to control transduced cells (CD74sc). Cells were plated at a density of 1,000 cells/well and exposed to 4-IPP one day after seeding. Cell cultures were processed for crystal violet staining after 3 days of drug exposure. In absence of 4-IPP treatment, CD74KD cell growth was reduced by 31% relative to CD74sc cell growth ($p<0.001$). Data are expressed relative to untreated CD74KD or CD74sc cells. Statistical analysis of differences between CD74KD and CD74sc cells was performed by Student's t-test.

cytometry analysis of the cell cycle, a 2-day exposure to 4-IPP led to a substantial cell accumulation in the G2/M phase of the cell cycle.

Expression of CD74 modulates SCCVII cell sensitivity to 4-IPP. MIF action on target cells notably depends on binding of its cognate receptor CD74 and the ensuing activation of downstream transduction cascade(s). Thus, we wondered whether a change of CD74 expression in SCCVII might influence their sensitivity to 4-IPP. The expression of CD74 was partially abrogated by lentiviral transduction of shRNA targeting the transcript (Fig. 5A). Fig. 5B illustrates the inhibition of growth induced by 4-IPP in CD74KD cells, as compared to control CD74sc cells. Blunting of CD74 expression clearly sensitizes cells to the cytostatic action of 4-IPP.

4-IPP reduces cell invasiveness. To evaluate whether MIF activity correlates with the invasiveness of SCCVII cells, we performed an *in vitro* cell invasion assay to determine the effect of 4-IPP on cell migration in Boyden chambers. 4-IPP at a concentration of 40 μM reduced the number of cells that migrated through the membrane as compared to untreated cells (Fig. 6A). The decrease of cell migration as a result of 4-IPP action was quantified by cell counting (Fig. 6B, $p=0.005$). This result suggests that MIF promotes cell migration through extracellular matrix and could contribute to tumor invasiveness *in vivo*.

Discussion

The prominent role of MIF as a proinflammatory cytokine makes it an attractive therapeutic target for the management of autoinflammatory disorders. Thus, there has been an intensive search for pharmacological compounds capable of blocking MIF activity. The design of new molecules and the high throughput screening for potential MIF inhibitors have

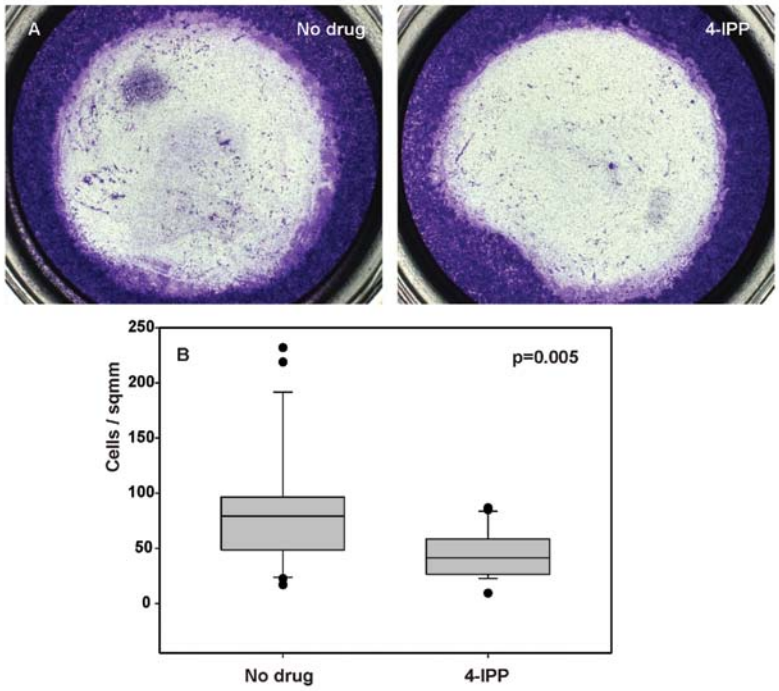


Figure 6. Influence of 4-IPP on the migration capacities of SCCVII cells. Inserts of Boyden chambers were seeded with SCCVII cells ($1.5x10^5$ cells per insert, four inserts per group). Membranes were stained 96 h after seeding and cells were removed from upper surface of the membranes by wiping with a moist cotton swab. (A) Typical fields showing the lower surface of inserts in absence or presence on 4-IPP (40 μM added to the lower chamber). (B) Cell densities on the lower surface, as determined by cell counting (see Materials and methods for details). Statistical analysis by Mann-Whitney test (n=23 measurements).

been facilitated by the fact that MIF possesses an enzymatic activity of D-dopachrome tautomerase. Albeit of unknown physiological significance, this tautomerase activity has been exploited as a validation tool for the identification of MIF inhibitors, since substances blocking this enzymatic activity would also be likely to interfere with MIF binding to its cognate receptor CD74. The first published report of a pharmacologically active MIF inhibitor described the properties of ISO-1 ((S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester). *In vitro*, ISO-1 was shown to abrogate MIF-mediated macrophage response to bacterial endotoxin, whereas *in vivo* it improved animal survival in a murine model of peritonitis-induced sepsis (30).

There is converging evidence that MIF contributes to tumor progression in various human cancers. In particular, recent observations of our group suggest an involvement of MIF in HNSCC. Indeed, immunohistochemistry reveals an increase of MIF immunostaining intensity during progression to neoplasia in hypopharyngeal (24), oral cavity (22) and laryngeal (23) squamous cell carcinomas. Higher serum levels were also found in patients with HNSCC, as compared to healthy individuals (23). These clinical observations were complemented by *in vitro* and *in vivo* studies on squamous cell carcinoma cell line SCCVII, where we showed that MIF knockdown decreases cell proliferation and motility and increases cell sensitivity to the anticancer drugs cisplatin and 5-fluorouracil (23).

Insofar as MIF seems to contribute to tumor progression, MIF inhibitors might be effective as anticancer drugs. Lending support to this assertion, ISO-1 has been reported to reduce the proliferation of glioblastoma multiforme cells *in vitro* (29) and inhibit the growth of tumor xenografts produced by the inoculation of prostate cancer cells (31) or colon carcinoma cells (20). In the present study, we investigated the impact of pharmacological MIF inhibition on the behaviour of SCCVII squamous carcinoma cells, since previous observations show that they are partially dependent on endogenous MIF for growth (23). As a test compound, we used 4-IPP (4-iodo-6-phenylpyrimidine), a suicide substrate which has been recently shown to exhibit 5-10 times more inhibitory potency than ISO-1 (26).

In the present study, preliminary experiments based on immunofluorescence staining revealed that SCCVII cells exhibit both MIF and CD74 immunoreactivities. Although similar studies have rarely been performed on tumor cells, MIF and CD74 have been previously shown to be co-expressed in non-small cell lung cancer (32). The intracellular pattern of MIF immunofluorescence in SCCVII cells was reminiscent of that reported for macrophages (33). As could be expected for a cell surface receptor, the distribution of immunoreactive CD74 markedly differed from that of MIF. Assuming that MIF can be secreted by SCCVII cells, the simultaneous expression of the cytokine and its receptor implies the possibility of autocrine stimulation.

The finding that 4-IPP treatment resulted in a decrease of MIF immunofluorescence intensity in SCCVII cells was at first sightly surprising since MIF inhibition has not been reported to decrease the expression of the cytokine in treated cells. As a matter of fact, ISO-1 has been observed to enhance rather than reduce MIF expression in glioblastoma multiforme cells (29). Further investigations relying on ELISA showed that 4-IPP actually alters MIF immunoreactivity, a finding consistent with a covalent modification of the latter. Similar observations on 4-IPP-induced alteration of MIF immunoreactivity have been reported previously (33).

Assessment of cell proliferation in presence of 4-IPP showed that the drug exerts a dose-dependent cytostatic effect on SCCVII cells, without evidence of apoptotic cells in treated cultures. CD74 knock-down definitely resulted in an increase of SCCVII cell sensitivity to 4-IPP. The additive effect of CD74 knock-down and 4-IPP suggests that MIF promotes SCCVII cell proliferation by interacting with CD74/CD44 and triggering downstream signalling events. Although the identity of the signalling pathway was not specifically investigated in the present study, MIF-induced enhancement of cell proliferation is generally assumed to occur via the activation of the ERK1/2 transduction cascade and the ensuing expression cell cycle proteins required for progression through G1/S (34,35). As shown recently with renal carcinoma cell lines, MIF also promotes cell proliferation by activating Src, which in turn phosphorylates and destabilizes cdk inhibitor $p27^{Kipl}$ (36). Insofar as MIF contributes to cell progression through G1/S phase, its inhibition would be expected to result in cell accumulation in G0/G1 phase. Such arrest in G0/G1 has indeed been demonstrated in HEK293 cells after MIF knockdown (37). Yet, cell cycle analysis by flow cytometry revealed that 4-IPP-treated SCCVII cells accumulate in G2/M. This observation raises the intriguing possibility that MIF might be involved in the control of mitosis by acting on G2/M regulators such as cdc25.

In the original report describing 4-IPP as a newly designed MIF inhibitor, the latter was shown to abrogate the migration of A549 lung carcinoma cells through collagen-coated membranes. Using a similar assay system, we noted that 4-IPP interferes with SCCVII cell migration across ECM-coated membranes. In a variety of neoplasms, MIF expression has been shown to correlate with tumor invasiveness/aggressivity. The mechanisms underlying MIF effects on tumor cell motility/invasiveness still remain a matter of debate and might depend on cell context. Observations on A549 cells point to involvement of the Rho GTPase Rac1 as an effector mediating MIF action on cell migration/invasiveness (38). A more recent study on chondrosarcoma cells suggests that MIF enhances cell migration via activation of the PI3K/Akt/NFκB signal transduction pathway and an increase of αvβ3 integrin expression (39).

The present study showed that pharmacological inhibition of MIF in squamous carcinoma cells resulted in impaired proliferation and invasiveness. HNSCC is not only a life-threatening disease, but its treatment can also be particularly debilitating. As revealed by a recent study of our group (23), MIF expression in HNSCC correlates with an unfavourable prognosis. Thus, the use of MIF inhibitors, particularly in conjunction with CD74 inhibition, might open new opportunities for target-directed treatment of these neoplasms.

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