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Inhibition of hyaluronan export from human fibroblasts by inhibitors of multidrug resistance transporters

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Abstract

In a previous report we described the export of hyaluronan from *Streptococcus pyogenes* by an ABC transporter. Extending these findings a sequence homology search against human proteins revealed a strong homology to the multidrug resistance transporter ABC-B (MDR-1) and ABC-C (MRP 5). Using several inhibitors directed against these and other transporters, a decreased hyaluronan production in cell culture as well as in hyaluronan synthase activity in purified membrane fractions was observed. The inhibitory capacity (IC_{50} concentrations) was compared the with reported IC_{50}- or the K_i-concentrations for individual transporters. These analyses revealed that hyaluronan is synthesized within the cytoplasm of mammalian cells and actively secreted into the pericellular space by energy dependent transport proteins. While inhibition of several transport proteins resulted in a decrease of hyaluronan export, inhibition of the MRP5 transporter was the most effective one to decrease hyaluronan in the cell culture supernatant indicating that hyaluronan export is one physiological role of this transport protein.

Keywords: Hyaluronan synthesis; Multidrug resistance; MRP5; ABC transporter; Plasma membranes; Extracellular matrix

1. Introduction

Hyaluronan is a large glycosaminoglycan that is abundantly present in the extracellular matrix, into which it is mainly secreted by fibroblasts, however, other cells may also contribute to its secretion. Hyaluronan plays an active role in regulating key cell behavior including random motility, chemotaxis, invasion, proliferation, shape, and metabolic reactions [1]. Many of these processes are fundamental for human diseases including cancer metastases, fight against infection and arthritis. For novel treatment strategies of these diseases an understanding of the mechanism of hyaluronan biosynthesis and its modulation including inhibition is vital. Hyaluronan biosynthesis proceeds by alternate transfer of the precursor nucleotide sugars UDP–GlcA and UDP–GlcNac at the reducing end at the inner face of the plasma membrane, from where the growing hyaluronan chain is exported directly into extracellular matrix [2–4]. The export was originally thought to be performed by the synthase itself in Streptococci [5] as well as in vertebrate cells [6]. However, we recently discovered that hyaluronan is exported through the protoblast membrane of Streptococci by an ABC transporter [60]. In this study, we describe that the streptococcal hyaluronan transporter has structural and functional homology to human multidrug resistance transporter.

Multidrug resistance transporters belong to the largest family of proteins, the ATP binding cassettes (ABC) transporters that are responsible for transport many compounds through cell membranes [7]. Although several substrates have been suggested as natural targets for multidrug resistance transporters including cholesterol for MDR-transporter and organic acids, cyclic nucleotides, and leukotriene for MRP-transporter, it still remains questionable, whether these substrates are really physiological ones. Because of their clinical significance, many inhibi-

Abbreviations: MDR, multidrug resistance; MRP, multidrug resistance associated protein

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tors for ABC-transporters have become available [7–11]. In addition to their intended clinical use, inhibition of transport proteins by a set of inhibitors that discriminate between different transport proteins is widely used to identify the transporter for a defined substrate [7,12,13].

The first inhibitors of hyaluronan synthesis were periodate oxidized nucleotide sugars that acted irreversibly as suicide inhibitors of the enzyme [14]. Because they had to be introduced into the cells by osmotic lysis of pinocytotic vesicles, they could only be used in cell cultures. The mode of action of other inhibitors such as vesnarinon [15] or 4-methylumbelliferone is not clear so far and they have not been widely used [16,17]. In this report, we describe a set of well-known drugs that inhibit hyaluronan synthesis by interfering with multidrug resistance transporters.

2. Materials and methods

2.1. Materials

Valspodar was a kind gift from Novartis. Other chemicals were from Sigma Chemical Co.

2.2. Cells and cell culture

Human fibroblasts were grown in suspension culture in Dulbecco's modified Eagles medium supplemented with streptomycin/penicillin (100 U of each/ml), kanamycin (100 U/ml) and 10% fetal calf serum. Cell proliferation was determined by cell counting after trypsinisation 3 days after seeding.

2.3. Hyaluronan synthase activity

Cells from five culture flasks (180 cm^2 growth area) were washed with cold phosphate buffered saline (PBS), harvested with the aid of a rubber policeman, sedimented at 1500 × *g* for 5 min and suspended in 30 ml of ice-cold PBS. The cells were transferred into a Parr-cell disruption bomb, exposed to a nitrogen pressure of 900 psi for 15 min and disrupted by nitrogen cavitation [18] and the particulate fraction was obtained by centrifugation at 40000 × *g* for 20 min. The sediment was suspended in 50 mM TRIS-malonate pH 7.0 at a protein concentration of 200 μg/ml and were mixed with an equal volume of the substrate for hyaluronan synthesis that contained 8 μM UDP–[^{14}C]UDP–GlcA, 166 μM UDP–GlcNac, 4 mM dithiothreitol, 20 mM $MgCl_2$ in 50 mM TRIS-malonate pH 7.0 and incubated at 37 °C for 4 h in the presence of increasing concentrations of multidrug resistance inhibitors. Hyaluronan synthesis was stopped by adding a solution of 10% sodium dodecylsulfate (SDS) to a final concentration of 1%. The mixtures were applied to descending paper chromatography that was developed with ethanol/aliquots 1 M ammonium acetate pH 5.5 (13:7) as solvent. After 18 h the radioactivity of [^{14}C]hyaluronan at the origin was determined.

2.4. Hyaluronan production

Trypsinised fibroblasts were suspended in Dulbecco's medium at 10^5 cells/ml and 100 μl aliquots were transferred to a 96-well microtiter plate. The first row received 200 μl of the suspension and 20 μl of the multidrug resistance inhibitors dissolved in DMSO at concentrations of 4 mM. A serial dilution to the inhibitors was established by transfer of 100 μl aliquots from the first row to the following rows. All experiments were performed in duplicates. The last row did not receive any inhibitor and served as control. Prior to these experiments it was established that the DMSO concentration used did not influence hyaluronan production or cell proliferation. The cells were incubated for 2 days at 37 °C and aliquots (5 and 20 μl) of the culture medium were used for measurement of the hyaluronan concentration in the cell culture medium by an ELISA [19]. Briefly, the wells of a 96-well Covalink-NH-microtiter plate (NUNC) were coated with 100 μl of a mixture of 100 mg/ml of hyaluronan (Healon®), 9.2 μg/ml of *N*-hydroxysuccinimide-3-sulfonic acid and 615 μl/ml of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide for 2 h at room temperature and overnight at 4 °C. The wells were washed three times with 2 M NaCl, 41 mM $MgSO_4$, 0.05% Tween-20 in 50 mM phosphate buffered saline pH 7.2 (buffer A) and once with 2 M NaCl, 41 mM $MgS0_4$, in phosphate buffered saline pH 7.2. Additional binding sites were blocked by incubation with 300 μl of 0.5% bovine serum albumin in phosphate buffered saline for 30 min at 37 °C. Calibration of the assay was performed with standard concentrations of hyaluronan ranging from 15 to 6000 ng/ml in equal volumes of culture medium as used for measurement of the cellular supernatants. A solution (50 μl) of the biotinylated hyaluronan binding fragment of aggrecan (Applied Bioligands Corporation, Winnipeg, Canada) in 1.5 M NaCl, 0.3 M guanidinium hydrochloride, 0.08% bovine serum albumin 0.02% $NaN_3$25 mM phosphate buffer pH 7.0 was preincubated with 50 μl of the standard hyaluronan solutions or cellular supernatants for 1 h at 37 °C. The mixtures were transferred to the hyaluronan-coated test plate and incubated for 1 h at 37 °C. The microtiter plate was washed three times with buffer A and incubated with 100 μl/well of a solution of streptavidin–horseraddish-peroxidase (Amersham) at a dilution of 1:100 in phosphate buffered saline, 0.1% Tween-20 for 30 min at room temperature. The plate was washed five times with buffer A and the colour was developed by incubation with a 100 μl/well of a solution of 5 mg *o*-phenylenediamine and 5 μl 30% H_2O_2 in 10 ml of 0.1 M citrate-phosphate buffer pH 5.3 for 25 min at room temperature. The adsorption was read at 490 nm. The concentrations in the samples were calculated from a logarithmic regression curve of the hyaluronan standard solutions.

3. Results

3.1. Homology search for eukaryotic hyaluronan transporters

The protein sequences of the streptococcal hyaluronan ABC-transporter Spy2194 and Spy2195 were used to search for homologous human sequences. The highest homology was found with ABCA, ABCB and ABCC transporters, three protein families of the large group of ABC-transporters. The phylogenetic relationship is shown in Fig. 1.

3.2. Effect on ABC-transport inhibitors on hyaluronan synthesis and proliferation of human skin fibroblasts

The structural homology of the streptococcal hyaluronan transporter with human multidrug resistant transporter could correspond well with identical functions. Because a wide range of multidrug transport inhibitors was available, a selected set of compounds were applied to human skin fibroblasts. The inhibitors were selected according to their discriminatory capacity to distinguish the different members multidrug resistance transporter. Three parameters were measured: (1) the amount of hyaluronan produced during growth into the culture supernatant; (2) the hyaluronan synthase activity in particulate membrane fractions to eliminate the possibility that inhibition of hyaluronan production in cell culture could be caused indirectly by cellular mediators; (3) the effect on cell proliferation, because hyaluronan synthesis is also required for detachment during mitosis and growth of the human HT1080 cell line [14].

The concentration dependency for 12 different inhibitors is shown in Fig. 2 and the IC_{50}-concentrations deduced from these results are summarized in Table 1. Several conclusions can be drawn from these results. Some inhibitors decreased not only hyaluronan production but also the synthase activity at concentrations that were in the same range usual for the transport inhibition of other known multidrug resistance transporter substrates. This finding indicated that a transporter function was required for hyaluronan export from human fibroblasts and this transporter was a member of the multidrug resistance transporter family. The direct inhibition of the synthase activity eliminated any interference from intracellular metabolites and this observation indicated that the hyaluronan synthase activity and export were coordinated.

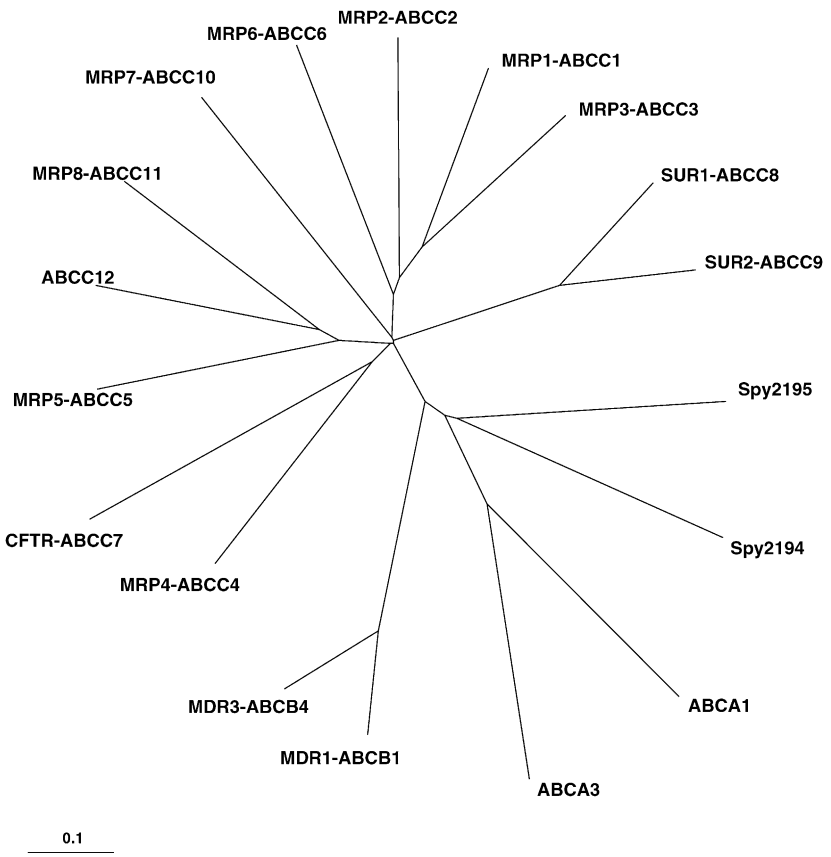

Fig. 1. The phylogenetic relationship of the streptococcal hyaluronan transporters Spy2194 and Spy2195 with two members of the human ABCA, ABCB and 12 members of the ABCC subfamily were aligned with the CLUSTALW program. The designations are given as names and symbols. The distance measure is given in substitutions per amino acid.

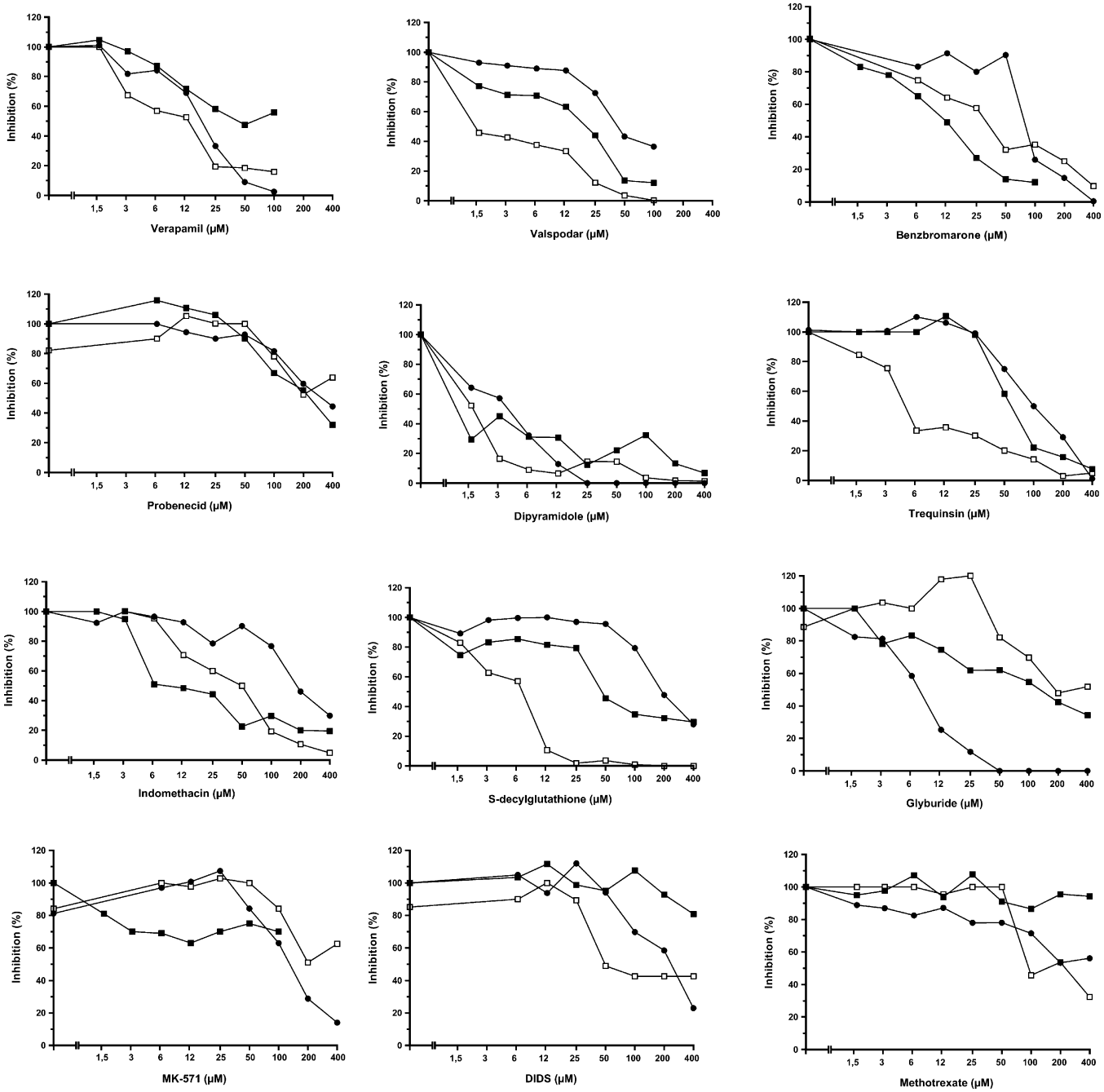

Fig. 2. Effect of ABC transporter inhibitors on the hyaluronan synthase activity (■), hyaluronan production (□), and proliferation (●) of human skin fibroblasts.

These inhibitors have a specific profile for certain members of the ABC transporter family and the published inhibitory concentrations (IC_{50} or K_i) are included in Table 1. A comparison of known inhibitory concentrations with that for the hyaluronan secretory activity could indicate which transporter was most likely for hyaluronan export. Thus, the inhibitors can be broadly arranged into two groups (Table 1): group 1 contains those inhibitors that decreased hyaluronan synthase activity at concentrations comparable with other secreted drugs and group 2 contains those that inhibited hyaluronan synthase activity at higher concentrations or not at all. Some inhibitors of group 1A such as verapamil and valspodar block a broad spectrum of transporter proteins and inhibited also hyaluronan synthesis indicating that the transporter belonged to the MDR- or MRP- family, which are both blocked by these.

The inhibitors of group 1B, indomethacin and the organic anion transport inhibitors benzbromarone and probenecid, in contrast preferentially inhibit MRP- and not MDR-transporters. As the latter three inhibited hyaluronan transport, a transport across the membrane by a member of the MDR-family appears unlikely, as the

Table 1
Inhibitory concentrations (IC_{50}, μM) of selected drugs for proliferation, hyaluronan synthase activity, and hyaluronan production of human fibroblasts and comparison with cited inhibitory concentrations (IC_{50} or K_i, μM) for the individual ABC transporters

Group	Inhibitor	Proliferation	HA production	HA synthase activity	Cited inhibitory concentrations							
					ABCA	MDR1	MRP1	MRP2	MRP3	MRP4	MRP5	MRP7
1A	Verapamil	20	15	~50		2–5				30	25	
1A	Valspodar	45	15	20		0.75	27	28.9		10		
1B	Benzbromarone	75	30	12		>800	4			150	150; <5	
1B	Probenecid	200	200	200		>2000	100–200			300	200	Weak
1B	Indomethacin	200	50	10		>800	10–20;50			20–50;5	>100	
1C	Dipyramidole	4	15	~1						2	10; 30	
1C	Trequinsin	100	6	60						10	0.24; 30	~100
1C	*S*-decylglutathione	190	7	40			37	Good inhibitor	Low affinity	Good inhibitor	Good inhibitor	
2	Glyburide	7	~400	120	5	100	10–50			1.9		
2	MK-571	150	>400	>100		0.11; 0.6	469; 13.1			1	No inhibition	~30
2	DIDS	250	50	>400	0.84		150–300			150–300		
2	Methotrexate	~200	~200	>400			Substrate	Substrate	Substrate	Substrate	No substrate	

members of the MDR family are not blocked by these inhibitors. The inhibitors of group 1C block MRP transporters differentially and can discriminate them. Dipyramidole is an effective inhibitor of MRP1, MRP4 and MRP5 and also of hyaluronan transport. Therefore one of these three transporters is a likely hyaluronan transporter. Trequinsin, a potent inhibitor of MRP4 and MRP5 transport, was also a good inhibitor of hyaluronan transport indicating that one or both of these transporters are likely hyaluronan transporters as well. *S*-decylglutathione, an inhibitor of all MRP-, but the MRP3-transporter, was a very good inhibitor of hyaluronan transport, hence the MRP3 transporter can be excluded as a hyaluronan transporter. Glyburide, an efficient inhibitor of ABC-A and MRP4 transporters, only inhibits at concentrations above 50 μM and the inhibition was not more than 50% even at 400 μM, thus ABCA1 and MRP4 transporter proteins can be excluded as likely candidates for transporting hyaluronan. MK-571 and methotrexate are good inhibitors for MRP1-4 and MRP7, but not for MRP5, both did not inhibit hyaluronan synthesis indicating again that the MRP1-4 and MRP7 transporters were unlikely candidates. DIDS preferentially inhibits ABC-A and the transport of inorganic anions and has low specificity for MRP transporters. It inhibited hyaluronan transport partially at relatively high concentrations, again excluding the ABC-A transporters and making the MRP transporters more likely candidates. All these data are compatible with hyaluronan transport preferentially by MRP5.

3.3. Effect on ABC-transport inhibitors on hyaluronan synthesis and proliferation of human synovial fibroblasts

The decrease of hyaluronan production possibly may also cause cytotoxic effects of the inhibitors on the cells itself. Therefore, we analyzed the effect of these drugs on human synovial fibroblasts, because these cells grow independently of hyaluronan synthesis (unpublished observation). Fig. 3 shows that the amount of hyaluronan in the culture supernatant was again inhibited in a concentration dependent manner. Verapamil inhibited cell growth, whereas valspodar decreased cell proliferation slightly, indicating that at least valspodar acted primarily on hyaluronan production.

4. Discussion

The export of hyaluronan from the synthase through the plasma membrane into the extracellular matrix by multidrug-resistance transporter explains many so far unresolved enigmas of hyaluronan synthesis. The presence of intracellular hyaluronan under certain growth conditions has so far been explained by cellular uptake [20,21]. However, this hypothesis could not satisfactorily explain how intact hyaluronan could bypass the breakdown process in lysosomes. Similarly, intracellular hyaluronan binding proteins have been found, however, and no appropriate intracellular function had been proposed for them [22,23]. By demonstrating that membrane transporters block hyaluronan export through the plasma membrane, hyaluronan synthesis has now been assigned to take place in the cytoplasm, which would resolve both above-mentioned problems.

Comparing the known IC_{50} or K_i-concentrations for a set of multidrug-resistance inhibitors with the inhibitory concentrations of hyaluronan synthesis suggested that MRP5 is the principle hyaluronan transporter in human fibroblasts.

The drugs verapamil and valspodar have rather broad transporter blocking specificities, as they inhibit MDR- as well as MRP-transporters. Verapamil is a licensed calcium channel blocker clinically used to treat arhythmias, in

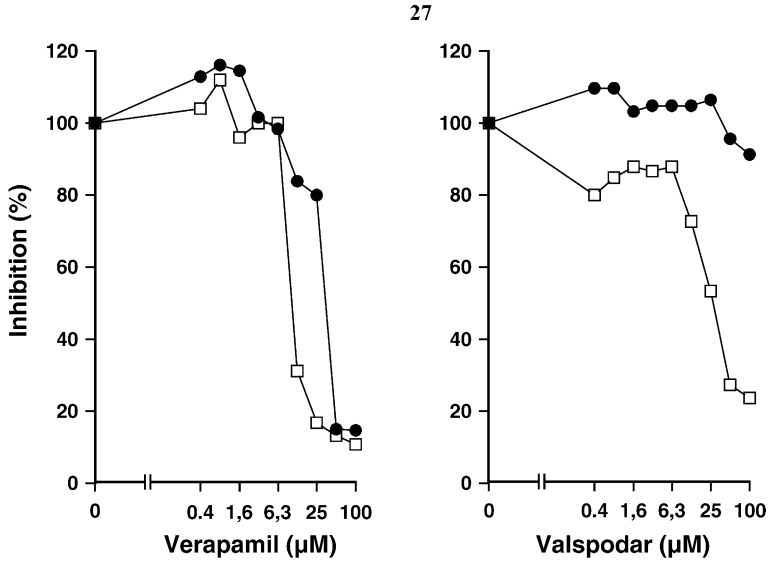

Fig. 3. Effect of verapamil and valsopodar on hyaluronan production (□) and proliferation (●) of human synovial fibroblasts.

addition to this function it also inhibits MDR1 with a K_i = 120 μM [24]. The IC_{50} concentrations for MDR1 and MRP have been reported be 2–5 μM and 4–8 μM, respectively [8]. It also inhibited the MRP4 mediated export of methotrexate with an IC_{50} ~ 30 μM [25]. It inhibits the export of fluorescein diacetate at a concentration of 25 μM by MRP5 [26].

Valspodar (PSC833) is a cyclosporin derivative with low toxicity and inhibited the MDR1 mediated transport of rhodamine with a K_i= 0.75 μM [27] and transport of leukotriene C4 by MRP1 with a K_i = 27 μM [28] and by MRP2 with a K_i = 28.9 μM [29]. It also inhibited the MRP4 mediated export of methotrexate with an IC_{50} ~ 10 μM [25]. Verapamil and valspodar inhibited with IC_{50} of about 50 and 15 μM, respectively. Thus members of the MDR and MRP family are required for hyaluronan export.

Benzbromarone and Probenecid are general inhibitors for organic anions that block reasorption of uric acid in the epithelia of kidney tubules. Benzbromarone is a general inhibitor of organic anion transporters that inhibits MRP1 with IC_{50} = 4 μM, both MRP4 and MRP5 with IC_{50} = 150 μM and MDR1 with IC_{50} > 800 μM [8]. Another study reported an IC_{50} < 5 μM for the MRP5 mediated transport of *S*-(2,4-dinitrophenyl)glutathione [30]. The 9-(2-phosphonomethoxyethyl)adenine (PMEA) transport by MRP4 or MRP5 was inhibited with IC_{50} = 150 μM [31]. It has no specificity for MDR transporters and was a very effective inhibitor of hyaluronan transport with an IC_{50} ~ 25 μM. This excluded the MDR transporter as hyaluronan transporter.

Probenecid preferentially inhibits MRP transporters at rather high concentrations of IC_{50} = 500–800 μM, whereas it has almost no inhibitory capacity towards MDR1 (IC_{50} > 2000 μM) [8]. Other reports measured its action on the MRP transporters of human erythrocytes that do not express substantial amounts of MDR1 and found an IC_{50} = 100–200 μM [32,33]. It also inhibited the MRP4 mediated export of methotrexate with an IC_{50} ~ 300 μM [25]. It inhibited the MRP5 mediated transport of cGMP at a concentration of 50 μM by 68% [34]. The 9-(2-phosphonomethoxyethyl)adenine (PMEA, an anti-HIV-drug) transport by MRP4 was very resistant to inhibition with an IC_{50} = 2300 μM, but sensitive by MRP5 with an IC_{50}=200 μM [31]. It inhibits the export of the fluorescent dye fluorescein diacetate at a concentration of 1 mM by MRP5 [26]. It was a weak inhibitor of the 17β-glucuronosyl-estradiol export by MRP7 [35]. Thus probenecid is a poor inhibitor for MDR1 and MRP4 and a rather good inhibitor for MRP5. Its inhibitory concentration for hyaluronan transport falls into a similar range as for MRP5.

Dipyramidol and Trequinsin are phosphodiesterase inhibitors. Dipyramidole is a vasodilator that is used to decrease the resistance coronary arteries. It inhibited the 9-(2-phosphonomethoxyethyl)adenine (PMEA) transport by MRP4 with an IC_{50} = 2 μM, and by MRP5 with an IC_{50} = 30 μM [31]. Another study reported an IC_{50} > 10 μM for the MRP5 mediated transport of *S*-(2,4-dinitrophenyl)glutathione [30]. Dipyramidole is an effective inhibitor of MRP1, MRP4 and MRP5 and of hyaluronan transport. Therefore one of these transporters is a likely hyaluronan transporter.

Trequinsin, a potent phosphodiesterase inhibitor, inhibited the MRP4 mediated export of methotrexate with an IC_{50} ~ 10 μM [25]. It inhibits the MRP5 mediated transport of cGMP with a K_i = 0.24 μM and of cAMP with a K_i = 0.38 μM [34], but inhibits the export of 17β-glucuronosyl-estradiol by MRP7 only moderately at 100 μM by 44% [35]. The 9-(2-phosphonomethoxyethyl)adenine (PMEA) transport by MRP4 was inhibited with an IC_{50} = 10 μM,

and by MRP5 with an IC_{50} = 30 μM [31]. Trequinsin, a potent inhibitor of MRP4 and MRP5 transport, was also a good inhibitor of hyaluronan transport. These experiments limit the likely hyaluronan transporters to MRP 4 and 5.

Indomethacin is a weak acid and is used in antirheumatic therapy as the first choice of nonsteriodal antirheumatic drugs (NSARD), because it inhibits the cyclooxygenase that is required for prostaglandin E2 synthesis, a mediator of pain and inflammation. It has already been shown that the indomethacin and its relative mefenamic acid inhibit hyaluronan synthesis in fibroblasts [36]. Indomethacin has a similar inhibitory spectrum as benzbromarone with IC_{50} > 800 μM for MDR1 and IC_{50} = 10–20 μM for the MRP transporter [8,32]. It inhibits the MRP4 mediated transport of cGMP with an IC_{50} ~ 20–50 μM [33] and the transport of 17β-glucuronosyl-estradiol by MRP4 with an IC_{50} ~ 5 μM and by MRP1 with an IC_{50} ~ 50 μM [37]. Another study reported an IC_{50} > 100 μM for the MRP5 mediated transport of *S*-(2,4-dinitrophenyl)glutathione [30]. Indomethacin is an inhibitor of all members of the MRP transporter family, but it does not inhibit any member of the MDR transporter family, and as it acts on the former transporter family showed a very good inhibition of hyaluronan synthesis. This finding again excluded the MDR transporter family as a hyaluronan transporter.

S-decylglutathione inhibited the leukotriene export by MRP1 with IC_{50} = 37 μM [38]. Simultaneously it stimulated the ATPase activity of the nucleotide binding domains of MRP1 (allocrites) [39]. *S*-decylglutathione, an inhibitor of transport of glutathione conjugates by MRP1, MRP2, MRP4 and MRP5 with low affinity for MRP3, was a very good inhibitor of hyaluronan transport, hence excluding the MRP3 transporter for hyaluronan.

Glyburide (glibenclamide) is a typical inhibitor of ABCA1 transporter that inhibits the secretion of macrophage inhibitory factor with IC_{50} concentrations of about 5 μM [40]. However, it also inhibited the export calcein (a fluorescent anionic dye substrate) by MRP1 in the concentration range of 10–50 μM [41] and the MDR1 mediated export of colchicine at a concentration of about 100 μM, because it is a substrate for MDR1 itself [42]. It is a very effective inhibitor of the MRP4 mediated transport of cGMP in human erythrocytes with an IC_{50} = 1.9 μM [33]. Glyburide, an efficient inhibitor of ABC-A and MRP4, only inhibits hyaluronan secretion at concentrations above 50 μM and the inhibition did not exceed 50% even at 400 μM. From these data we excluded the ABCA1 and MRP4 transporter as likely candidates.

MK-571, a leukotriene analogue, inhibits preferentially MRP1 with K_i = 0.6 μM [28] and has an IC_{50} = 0.11 μM for leukotriene export [38]. Its inhibitory activity is much lower towards MRP2 with an IC_{50} = 469 μM [43]. Another study reported a K_i = 13.1 μM for the MRP2 mediated export of leukotriene C4 [29]. It inhibited the 17β-glucuronosyl-estradiol export by MRP7 at 30 μM by 42 % [35]. It also inhibited the MRP4 mediated export of methotrexate with an IC_{50} ~ 1 μM [25]. It has no inhibitory effect on the MRP5 mediated transport of cGMP at concentrations up to 50 μM. The 9-(2-phosphonomethoxyethyl)adenine (PMEA) transport by MRP4 was sensitive to inhibition with an IC_{50} = 10 μM, but relatively resistant by MRP5 with an IC_{50} = 40 μM [31]. MK-571 is a good inhibitor for MRP1-4 and MRP7, but not for MRP5. This drug inhibited hyaluronan production only partially at concentrations above 100 μM. If an inhibitor selected from MRP1-4 and MRP7 were responsible for hyaluronan transport, a more efficient inhibition would be expected. But this was not the case. Therefore the MRP1-4 and MRP7 transporters do not appear to be preferred hyaluronan transporters in human fibroblasts.

DIDS (4,4-diisothiocyanato-stilbene-2,2-disulphonate) is also an inhibitor of anion transport, but it prefers inorganic anion transporters such as the chloride channel with a K_i = 0.84 μM [44], however it has also low inhibitor action on the MRP transporters of human erythrocytes (IC_{50} = 150–300 μM) [32]. DIDS preferentially inhibits ABC-A and inorganic anions and has low specificity for MRP transporters. It inhibited hyaluronan transport partially at relatively high concentrations, again excluding the ABC-A transporters and making MRP transporters more likely candidates for hyaluronan transport across the cell membrane.

Methotrexate is a substrate for MRP1, MRP2, MRP3 and MRP4 [45], but not for MRP5 [46]. It did not inhibit the interleukin 1 stimulated secretory activities of cultured human synovial fibroblasts [47]. It also did not inhibit the hyaluronan synthase activity making MRP1, MRP2, MRP3 and MRP4 unlikely as hyaluronan exporters.

MRP5 analysis is still in its infancy. It is an organic anion transporter and most closely related to MRP4. MRP5 transports the fluorescent dye fluorescein diacetate [34], cAMP and cGMP and of glutathione conjugated compounds such as *S*-(2,4-dinitrophenyl)glutathione [30], but not leukotriene C4, 17β-glucuronosyl-estradiol, calcein, GSSG, and PGE1 or PGE2 [37]. It can be expressed in several splicing variants [48]. Knockout mice do not display obvious abnormalities, despite the fact that MRP5 is expressed in all tissues analyzed thus far [26,49].

From the inhibitory profile of the drugs it can be concluded that MRP5 the most likely hyaluronan transporter of human fibroblasts. This conclusion does not imply that MRP5 transports hyaluronan exclusively, as MRP5 knockout mice are viable thus indicating that alternative transport systems within cells can compensate for the lack of MRP5. These transporters could be ABCC11 or ABCC12 due to their close phylogenetic relationship [50]. Hyaluronan deficiency would be expected to be incompatible with life, because it is required for cell differentiation immediately after fertilization [51] as well as for fibroblasts proliferation [14], both observations highlighted by the fact that hyaluronan deficient knockout mice die at the stage 10.5 [52]. Hence other channels must mediate hyaluronan

secretion in MRP5 knockout mice. In their publication, Reid et al. ended their discussion on the function of MRP4 and MRP5 with the statement: ''For MRP5, a high-affinity substrate remains to be found'' [31], which we herewith present.

As most tissues contain hyaluronan, the ubiquitous tissue distribution of MRP5 also supports the hypothesis that it is a hyaluronan transporter, while most of the other MRP transporters such as MRP2, MRP3 and MRP4 have a much more restricted tissue distribution making them unlikely candidates for hyaluronan transport. The broad expression of MRP5 also extends to cartilage, because MRP5 mRNA could be detected in human chondrosarcoma cells (own unpublished observation). It is interesting that MRP5 is present in the brain [49] and even in various regions of the brain [26] matching the observation that hyaluronan is synthesized in the various regions of the brain as well [53]. The only other MRP family member known to reside in the brain is MRP1, which is restricted to the choroid plexus, making this member of the MRP family an unlikely transporter of hyaluronan. MRP5 is also expressed in cardiac muscle cells of human heart and is enhanced under ischemic conditions [54], which correlates well with the observation that hyaluronan synthesis is increased in myocardial infarction [55]. Thus, the tissue distribution of MRP5 and hyaluronan are in broad agreement with the proposed function of MRP5 as a hyaluronan transporter.

Our conclusion that a multidrug resistant transporter is involved in hyaluronan export is further supported by the observation that increased hyaluronan production induced resistance in drug-sensitive tumor cells [56]. The mechanism of this inhibition could simply be explained by competition of two substrates for the transporter.

Our observation that both hyaluronan synthase and transport were inhibited simultaneously implies that these activities were coordinated within the plasma membranes in such a way that growing hyaluronan chains exerted a feedback inhibition on the synthase. This phenomenon has been described previously [57,58]. The results also showed that inhibition of hyaluronan synthesis reduced cell proliferation and verified previous studies [14]. A similar coordination of synthesis and export has previously been observed for polysialic acid [59].

Finally, the our discovery of drugs that are used for other diseases for inhibition of hyaluronan synthesis may open novel ways for treatment of diseases that are characterized by HA overproduction such as edema formation after injuries, inflammation and metastasis.

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