Investigation of Aminomethyl Indole Derivatives as Hyaluronidase Inhibitors

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Hyaluronidase inhibitors are of potential therapeutic value for the treatment of a variety of diseases, such as cancer, arthrosis, or bacterial infections. Potent and selective hyaluronidase inhibitors are not known so far, and current approaches to the development of hyaluronidase inhibitors are limited. Elevated levels of hyaluronan (HA) are connected with most malignant tumours. Therefore, the search for drugs modulating the hyaluronidase activity became very important. In the present study, a new series of aminomethyl indole derivatives (AMIDs) were tested for inhibition of bovine testes hyaluronidase (BTH). *In vitro* assays were performed using stains-all at pH 7 and Morgan-Elson reaction at pH 3.5. Among the AMIDs, 3-[(4-methylpiperazin-1-yl)methyl]-5-phenyl-1*H*-indole (**9**) was found to be active with 23% inhibition at 50 μM and pH 7. All the other inhibitors showed less activity at pH 3.5 and pH 7. These activity results demonstrated that compounds with phenyl substitution at position 5 have higher activity. The results confirmed that more lipophilic compounds have better inhibition against the hyaluronidase enzyme.

Key words: Indole Derivatives, Inhibitors of Hyaluronidases, Cancer

Introduction

Hyaluronan (HA) plays an important role in tissue architecture (Laurent and Fraser, 1992). Furthermore, HA is important for cell proliferation, cell migration, and cell growth as well as the metastasis of tumour cells (Laurent *et al*., 1996). Recent studies have shown that most malignant tumours have elevated levels of HA (Boregowda *et al*., 2007). Human breast, lung, prostate, ovarian, nephroblastomas and colon cancer are considered to enrich with HA. In these tumours, HA may support tumour growth by stimulating anchorage-independent growth and proliferation of tumour cells (Grish and Kemparaju, 2007; Liu, 1996). Therefore, hyaluronidase inhibitors are of potential therapeutic value for the treatment of a variety of diseases, such as cancer, arthrosis, or bacterial infections. Inhibitors of hyaluronidases also represent a completely new group of cytostatic drugs, contraceptives, and antiarthrotic agents (Maingonnat *et al*., 1999). Since current approaches to the development of hyaluronidase inhibitors have been limited so far, several hyaluronidase inhibitors have been studied in order to find potent and selective inhibitors. Different chemical groups of hyaluronidase inhibitors such as proteins, glycosaminoglycans, polysaccharides, fatty acids, lanostanoids, antibiotics, antinematods, synthetic organic compounds, and plant-derived bioactive components, such as alkaloids, antioxidants, polyphenols, flavonoids, terpenoids, and anti-inflammatory drugs (Grish *et al*., 2009), have been recently reported. Among them, flavonoids (Grish and Kemparaju, 2007) and *O*-sulfonated glycosaminoglycans (Toida *et al*., 1999), apigenin (Trochon *et al*., 2000), *cis*-hinokiresinol (Jeong *et al*., 2003), marimastat (Gore *et al*., 1996), and SU6668 (Laird *et al*., 2002) did not show prominent bovine testes hyaluronidase (BTH) inhibitory effects whereas Vcpal (6-palmitoyl-L-ascorbic acid) strongly inhibited BTH with an IC_{50} value of 56.5 μM (Botzki *et al*., 2004). Several synthetic indole, benzoxazole, and benzimidazole derivatives were studied as hyaluronidase inhibitors (Fig. 1). Based on these studies, several mono- and diacylated benzimidazole-2-thiones were synthesized, and 1-acetyl-3-hexanoylbenzimidazole-2-thione was found to be a strong inhibitor of hyaluronidase (Braun, 2005). 1-Decyl-2-(4-sulfamoyloxyphenyl)-

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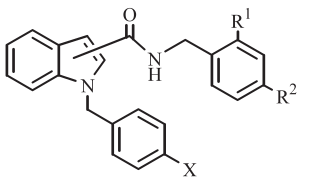
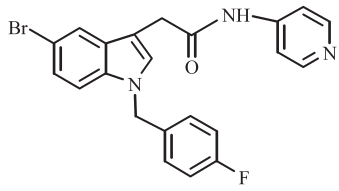
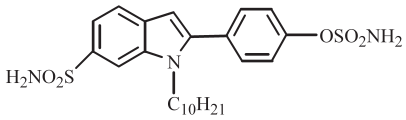


X= H, F R^1, R^2= H, 4-Cl, 4-F, 2,4-Cl, 2,4-F

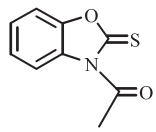
Indole-2- and -3-carboxamide derivatives



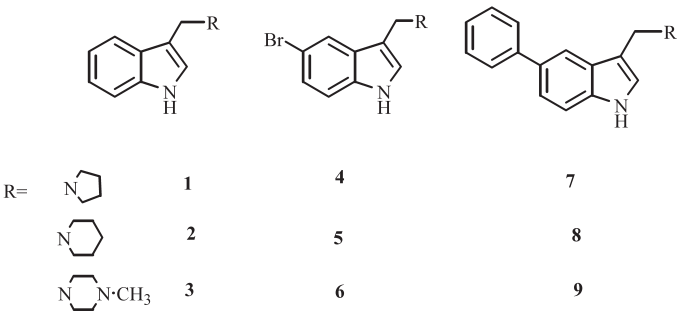
N-(Pyridin-4-yl)-[5-bromo-1-(4-fluorobenzyl) indol-3-yl] acetamide



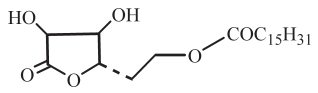
1-Decyl-2-(4-sulfamoyloxyphenyl)-1*H*-indol-6-yl sulfamate



N-Substituted benzoxazole-2-thione



Aminomethyl indole derivatives



Vcpal

Fig. 1. Compounds as hyaluronidase inhibitors.

1*H*-indol-6-yl sulfamate and *N*-substituted benzoxazole-2-thione derivatives (Fig. 1) showed an IC_{50} value of 11 μM and 15–260 μM, respectively (Rigden *et al.*, 2006; Botzki *et al.*, 2005). In addition, 1,3-diacetylbenzimidazole-2-thione was also tested, and the IC_{50} values of 160 μM at pH 5 and 5 μM at pH 7.4 were obtained (Rigden *et al.*, 2006). Another study showed that benzoyl phenyl benzoate derivatives inhibited the hyaluronidase activity of *Naja melanoleuca*, *Naja naja*, *Vipera russellii*, and *Trimeresurus elegans* venoms (Khanum *et al.*, 2005). BTH inhibitory activities of several indole-2- and -3-carboxamide derivatives (Fig. 1) were investigated, and it was found that the most active compound, *N*-(4-fluorobenzyl)-[1-(4-fluorobenzyl)indol-3-yl] carboxamide, had an IC_{50} value of the 26 μM at pH 7 (Olgen *et al.*, 2007). The IC_{50} value of the reference inhibitor Vcpal (Fig. 1) was measured as 8.36 μM under the same assay conditions. In recent studies, several indole and benzimidazole derivatives were identified as hyaluronidase inhibitors (Kaessler *et al.*, 2008). Among them, *N*-(pyridin-4-yl)-[5-bromo-1-(4-fluorobenzyl)indol-3-yl] acetamide (Fig. 1) was the most potent derivative with an IC_{50} value of 46 μM. Algul *et al.* (2008) studied several benzimidazole, benzothiazole, and indole derivatives, and 2-(4-hydroxyphenyl)-3-phenyl indole was found to be the most potent inhibitor at pH 7 and pH 3.5. Thus, the authors confirmed that indole deriv-

atives are more potent hyaluronidase inhibitors than benzimidazole and benzothiazole derivatives. To prove and affirm the role and importance of indole derivatives as hyaluronidase inhibitors, a new series of aminomethyl indole derivatives (AMIDs) were tested for the inhibition of BTH to find selective and potent inhibitors.

Experimental

Measurement of hyaluronidase activity

Hyaluronidase from bovine testes was purchased from Serva (Heidelberg, Germany), hyaluronic acid was purchased from Sigma Aldrich (Steinheim, Germany). Water was purified using a Milli-Q Biocel system.

Stains-all assay

An enzyme solution of 100 U/ml hyaluronidase was prepared from 3110 U/mg hyaluronidase powder with 50 mM sodium phosphate buffer at pH 7. As substrate solution, 2 mg hyaluronic acid were dissolved in 1 l water. All compounds, **1**–**9**, were prepared with DMSO to give stock solutions of 10 mM. 25 μM, 50 μM, and 100 μM inhibitor concentrations were obtained by adding inhibitor stock solution to the enzyme solution. The inhibitor/enzyme solution was incubated for 1 h at 37 °C. After incubating the substrate solution, 0.2 M phosphate buffer and water were mixed and spotted onto a microplate. The assay was started by adding the same amount of enzyme/inhibitor solution to the wells. While running, the plate was incubated at 37 °C. To measure the HA concentration, stains-all solution and water were added to the wells, and the absorption was monitored at 650 nm by a microplate reader (Mithras LB940, Berthold Technologies, Bad Wildbad, Germany). The activity of a positive control with pure DMSO instead of inhibitor solution was measured and set to 100%. A more detailed description of the assay procedure and used solutions has been published recently (Kaessler *et al.*, 2007). For investigating the influence of NaCl and bovine serum albumin (BSA) concentrations on BTH activity, enzyme solutions of 100 U/ml hyaluronidase containing different amounts of NaCl and BSA were prepared and used in the same way as the pure hyaluronidase solution. Negative controls without enzyme were used to eliminate possible interactions of stains-all solution with BSA or NaCl.

Morgan-Elson assay

An enzyme solution of 800 U/ml hyaluronidase was prepared from 3110 U/mg hyaluronidase powder with formate buffer containing 0.1 M sodium formate and 0.1 M NaCl at pH 3.5. The compounds, **1**–**9**, were dissolved to 10 mM in DMSO as above mentioned. 5 mg HA were dissolved in 1 l water to give the substrate solution. 25 μM, 50 μM, and 100 μM inhibitor concentrations were obtained by adding inhibitor stock solution to the enzyme solution. The inhibitor/enzyme solution was incubated for 1 h at 37 °C. After incubation 0.2 mg/ml BSA solution, incubation buffer, water, and inhibitor/enzyme solution were mixed, and the assay was started by adding substrate solution. While running, the mixture was incubated at 37 °C. For investigating the influence of different BSA concentrations on the BTH activity at pH 3.5, solutions with different protein concentrations were used in the same way as the original 0.2 mg/ml BSA solution. To measure the HA fragments concentration, several quota of the incubated solution were heated for 4.5 min at 100 °C after addition of a KOH solution. Subsequently, the solutions were transferred onto a microplate and stained using DMAB (dimethylaminobenzaldehyde). The absorption was measured at 590 nm using a Mithras LB940 microplate reader. The activity of a positive control with pure DMSO instead of inhibitor solution was set to 100%. A more detailed description of the assay procedure and used solutions has been published by us recently (Kaessler *et al.*, 2007). For both assays a negative control lacking hyaluronidase enzyme was measured. 6-Palmitoyl-L-ascorbic acid was used as a control compound in the same way as the other inhibitors. For compound activity measurement at pH 7 with the Morgan-Elson assay a phosphate-citrate buffer containing 0.1 M sodium citrate, 0.2 M sodium dihydrogenphosphate, and 0.1 M NaCl was used in the same way as the formate buffer at pH 3.5.

Results and Discussion

In the present study, a new series of AMIDs (Fig. 1) were tested for their hyaluronidase inhibitory capabilities at pH 7 and pH 3.5 using the recently developed stains-all and Morgan-Elson assay (Kaessler *et al.*, 2007). All mammalian hyaluronidases belong to an identical class

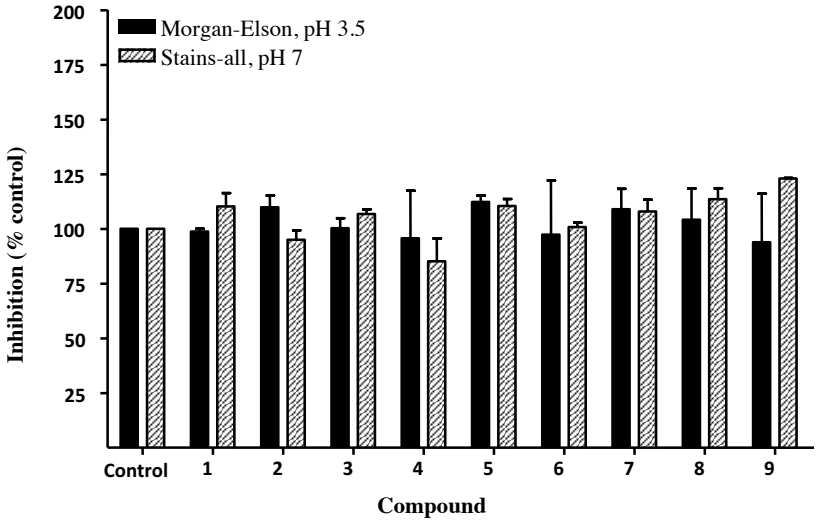


Fig. 2. Effects of the aminomethyl indole derivatives on the enzymatic activity of bovine testes hyaluronidase.

of enzymes catalyzing the degradation of HA by cleavage of the $\beta(1\rightarrow4)$-bonds between the disaccharide units. The most important human hyaluronidases are Hyal-1, Hyal-2, PH20, and BTH (Stern and Jedrzejas, 2006). Hyal-2, PH20, and BTH show high activities over a wide range starting at neutral pH to pH 3, whereas Hyal-1 is much more active at acidic pH than at neutral pH (Frost and Stern, 1997). Therefore, it is necessary to test the inhibition profile of potential inhibitor compounds and also to measure the inhibition at neutral as well as acidic pH values.

In the present investigation all assays were performed using BTH, which exhibits a homology of about 65% to PH20 and 40% to Hyal-1, at pH 7 and pH 3.5, as described earlier (Olgen *et al.*, 2007). Among the AMIDs, 3-[(4-methylpiperazin-1-yl)methyl]-5-phenyl-1*H*-indole (**9**) was the most active compound with 23% inhibition at 50 μM and pH 7 (Table I and Fig. 2). All the other inhibitors showed less activity at pH 3.5 and pH 7. These results revealed that an aminomethyl indole derivative having phenyl substitution at position 5 showed higher activity.

In our previous study, twenty *N*-substituted indole-2- and -3-carboxamide derivatives (Fig. 1) were tested for their capability of hyaluronidase inhibition at a concentration of 50 μM at pH 7 (Olgen *et al.*, 2007). Among them, nine inhibitors caused more than 50% inhibition with IC_{50} values ranging between 25 and 41 μM. It was reported that *para*-positions of benzamide should be chlorinated or fluorinated to obtain good inhibitory activity for both indole-2- and -3-carboxamide derivatives. The activity profiles of compounds substituted at positions 2 and 3 were found to be slightly different. It was also reported that compounds containing *N*-benzyl-substituted, mono-halogenated benzamide at position 2 had good activity while their congeners substituted at position 3 did not show any prominent activity on enzyme. Comparison of these previous and current results confirmed that more lipophilic compounds have better inhibition against hyaluronidase activity. In our previous study it was shown that the introduction of a *p*-fluoro benzyl ring at the indole nitrogen atom of mono-halogenated benz-

Table I. Inhibitory effects (% control) of aminomethyl indole derivatives on hyaluronidase activity at pH 3.5 and 7.

Compound	Morgan-Elson assay, pH 3.5	Stains-all assay, pH 7
1	–1.80	10.30
2	9.90	–5.00
3	0.30	6.80
4	–5.32	–4.80
5	12.33	10.50
6	–2.70	0.80
7	9.00	8.00
8	4.20	13.60
9	–7.10	23.00

amide derivatives exhibited good inhibitory effects (Olgen *et al.*, 2007). This result suggests that the fluoro substitution of the benzyl ring has a positive effect on the inhibition of hyaluronidase. This could be the explanation that AMIDs unsubstituted at the indole nitrogen atom have weak inhibitory effects. Another explanation for lack of activities of AMIDs could be, that the feature of a side chain at position 3 of indole is not suitable to fit in the active site as good as previously reported indole carboxamide derivatives, probably due to the shorter backbone and lipophilic or hydrophilic effects of substituents.

Recently, it was reported that halogen bonding interactions are responsible for different conformations in the active site of the molecules dealing with many factors that influence inhibitor recognition and binding, such as specificity surface and hydrophobic effect (Liu *et al.*, 2009). Among halogens, especially fluorine can improve other pharmacokinetic properties by means of influencing the p*K*a value, elevating the lipophilicity, and reducing the plasma protein binding (DiMango and Sun, 2006). Fluorine is also known to be able to form multi-polar interactions with several amino acid residues, responsible for the enhanced binding potency, and it is capable to enhance the metabolic stability (Hagmann, 2008). Since halogen substitutions are not be longer validate for AMIDs, which have weaker inhibitory potencies than halogenated indole-2- and -3-carboxamide derivatives, it can be concluded that lipophilic properties might be important for hyaluronidase inhibition. It was previously reported that HA activation and inhibition is pH-dependent and probably an effect of protonation (Kaessler *et al.*, 2008). The bonding of the protonated form of compounds can result in different behaviour to enzymatic access. Another possible explanation for lack of hyaluronidase activity could be a possible protonation of the AMIDs from nitrogen atoms (indole, pyrrole, pyrimidine, and piperazine ring nitrogen atoms). This protonation could cause positive charges, and this additional charge would then change the activating effect on HA degradation.

Acknowledgements

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