るMetがグルタミン合成酵素の活性サイトの入り口に位置していることに注目し，活性サイトにおいて機能発現や構造維持に重要なアミノ酸残基をラジカルによるダメージから守るスカベンジャーとしてMet残基が機能している可能性を示している[11]．好都合なことには，Metの酸化体であるメチオニンスルフォキシド（MSOX）は細胞内に存在するMSOX還元酵素により元のMetへ変換される[12]．したがって，Metは細胞内ではこのサイクルにより半永久的に抗酸化剤として働くことも可能である．

③サイトⅠに位置する残基（199Lys，214Trp，218Arg，242His）

サイトⅠへは，ワルファリン，フェニトイン，フロセミド及びグリベンクラミドをはじめとする多くの薬物が結合する．このサイトⅠは一次配列における199Lys～292Gluに存在しており，214Trpなどの疎水性アミノ酸残基からなる疎水領域に242Hisおよび199Lysといった陽電荷が局在している．事実，作製したHSA変異体を用いた検討結果から，199Lys，214Trp，242Hisのいずれもが薬物結合に深く係りわり合っていることを確認している[13]．また，214Trpは，HSA分子上，中心に位置することから，構造維持に必須なアミノ酸残基として機能している．加えて，Bhagavanらは，214Trp→Ala変異体を作製し，抗酸化能が約70%に低下したことから，214Trpを抗酸化アミノ酸残基として定義している[5]．最近，Trpの結合特性を活用してHSA製剤に大量に添加されているN-Acetyl-Trpが，抗酸化剤として機能していることが報告された[14]．すなわち，214Trpは，HSAの構造と機能を担う必須なアミノ酸残基として役割を果たしていると考えられる．また，家族性高チロキシン血症（FDH）はチロキシンの血中濃度が通常よりも著しく高い疾患であるが，この原因として218ArgのHisへの変異が明らかにされている．事実，Bhagavanらは218ArgのHisへの変異体を用いてチロキシンの結合性が約65倍増加することを見出している[15]．

④サイトⅡに位置する残基（410Arg，411Tyr）

著者らはケトプロフェンを用いた光親和性ラベル化法に加えて，部位特異的変異法を用いた実験結果から，サイトⅡに対する薬物結合には410Argのグアニジノ基に加え，411Tyrの水酸基とその芳香環も重要な役割を果たしていることを明らかにしている[16]．さらに，このサイトへは経腸栄養剤や脂肪乳剤に含まれる中鎖脂肪酸も強く結合しHSAの構造安定性を向上させることが知られている．この特性を活用してHSA製剤にはC_8のカプリル酸が安定化剤として大量に添加されている[14]．サイトⅡは上述した薬物結合能に加え，エステラーゼ様作用を有する．著者らは410Arg→Ala変異体，411Tyr→Ala変異体あるいは両者の二重変異体を用いた実験結果から，この酵素反応の活性中心が411Tyrの水酸基であることとともに，410Argがその反応性を高めていることを見出している[17]．また，菊川らは，*in vitro*においてウシ血清アルブミン（BSA）を過酸化水素により処理後，血中セリンプロテアーゼである酸化蛋白質分解酵素により，この酸化BSAを処理したところ，BSAの409Arg-410Tyr-411Thr近傍を特異的に切断することを報告している[18]．この結果を受けて，著者らは，BSAの切断部位に相当するHSAの410Arg→Ala，411Tyr→Ala変異体を用い，その体内動態をwild-typeと比較検討した結果，410Argの変異体においてのみ素早い消失を確認している．すなわち，変異による410Arg近傍のコンフォメーション変化が血中の酵素により切断された結果，消失の促進を引き起こしている可能性が考えられる．

2．遺伝子組換え型HSAを利用した医薬への応用

従来，HSAは生体分解性や血中滞留性に富んでいるため，動態特性上問題がある薬物のDDSにおいて好ましい担体として用いられてきた．高分子医薬に対し，アルブミンを担体として付与する場合，アルブミンが有するリガンド結合能を利用した非共有結合，あるいは両者を化学修飾によるいずれかの方法が用いられてきた．しかしながら，化学結合の場合，均一な結合体の調製が難しい上，両成分の生理活性や特性を保持させるためには，適切な架橋条件を見出す必要があるなどの問題点が指摘されている．最近，このような問題点を克服するため，アルブミンと蛋白性医薬品の遺伝子を融合させたfusion-proteinの開発が盛んに行われている．さらに，臓器指向性や，主薬の安定化向上を狙ったHSA自身のサイズ調節をはじめ，新規蛋白医薬品としてのアルブミンの医療への応用も試みられている．

1）チオール基（34Cys）を利用した医薬への応用

①一酸化窒素との相互作用

一酸化窒素（NO）やカプトプリル，ブシラミンといったSH含有薬物は34Cysに共有結合する．特に，血管弛緩因子であるNOは血液中でS-ニトロソ蛋白として運搬され，その8割がS-ニトロソHSAとして存在している[19,20]．S-ニトロソHSAになると，血管平滑筋を弛緩させたり，血小板凝集を抑制したり，アデニール・サイクレースを活性化させたりする．一酸化窒素（NO）が新しい生理活性物質として認識され，生体内NO結合メディエーター蛋白として，ヘモグロビン（Hb）やHSAがその対象として研究されている．虚血性疾患や臓器移植時などの病態時では，内因性のNO産生低下に伴い外因的なNOの補充が必要不可欠であることから，比較的長寿命で安定な新規NO補充療法の開発が望まれている．著者らは，NO導入効率改善のため，Cysを付加した410Arg→Cys変異体を作製し，これにNOを付加した新規S-ニトロソタンパクを作製した．そのNO導入効率は，wild-type-HSAの約4倍まで上昇し，その物性，生物活性及び体内動態特性において，いずれもwile-typeに比べて，S-ニトロソタンパクとして，より優れていることを明らかにしている[21]．今後，Cysを付加した組換え型S-NO-アルブミンの新規NO抱合運搬タンパク（NOトラフィックタンパク）としての応用開発の可能性が大いに期待される．

②34Cys架橋による酸素輸液への応用

血液型のない，感染の危険もない輸血に代わる新技術として，人工酸素運搬体の開発が盛んに行われている．土田らは，高濃度酸素輸液の調製を目指して，rHSAに疎水性の合成ヘム

"lipidheme"（ポルフィリン誘導体：Lh）を効率よく包摂させたアルブミン-ヘム複合体（rHSA-Lh）を作製している．このアルブミン-ヘムは自然界に存在しない全く新しい合成ヘム-蛋白質であるが，Hbと同様，酸素分圧に応じて酸素分子を結合解離することが可能であり，HSA1分子あたり８分子のLhを結合できることから，高濃度酸素輸液としての臨床応用が進められている．さらに，最近，rHSA二量体（$rHSA_2$）を^{34}Cysの架橋形成により作製し，二量体による最大16分子のLhが結合可能となっている．また，このrHSA₂におけるラットの体内動態特性を評価したところ，rHSAの約1.5倍の半減期を示し（Fig. 3.），更なる高濃度酸素輸液として開発が進められている[22]．

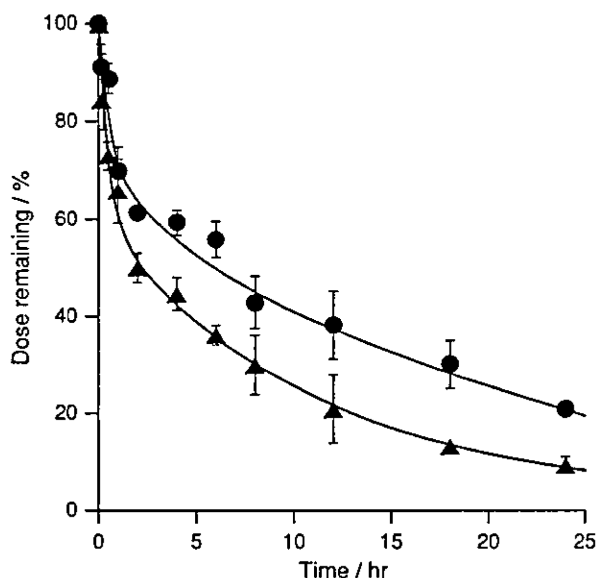

Fig. 3. Plasma levels of ^{125}I-rHSA monomer (△) and dimmer (○) (1.0× 10^7 cpm, 1.0 mg kg^{-1}) after intracardial administration into Wistar rats. All values are mean ± S.D. (n=3). (From Ref. 22)

③^{34}Cys架橋による効率的薬物輸送

麻薬性鎮痛薬であるDynorphin Aは，その早い消失のため，頻回投与による副作用が問題となっている．しかし，最近，Holmesらは，^{34}CysとDynorphin AをDrug Affinity Complex（DAC）システムにより１対１の割合で化学的に結合させ，血中滞留性の向上とともに，肝臓，腎臓や脳などへの組織移行を制限に伴う副作用の軽減に成功している[23]．現在，ConjuChem社において，第一相臨床試験が行われている．

２）HSAの血中滞留性を利用した薬物動態制御システム

①長期作用型インスリン

インスリンは糖尿病治療薬として汎用されているが，その糸球体濾過に伴う消失の促進による頻回投与が問題となっている．Markussenらは，インスリンの^{29}Lysを長鎖脂肪酸の一つであるミリスチン酸で修飾し，血液中でHSAと結合可能なアシル化インスリンを設計している[24]．HSAとの結合はアシル化インスリンの腎臓や肝臓からの消失を抑制し，血中滞留性を改善することから長時間型インスリンへの応用が期待されている．

②長期作用型新規リウマチ性治療薬

Fiehnらは，リウマチ様関節炎の新しい治療薬へのアプローチとしてアルブミンのLys残基をN, N'-dicyclohexyl-carbodiimide（DCC）及びN-hydroxysuccinimide（HSI）により架橋したメトトレキサート（ＭＴＸ）-アルブミン複合体を設計した[25]．従来，MTXは，その低分子量により，糸球体濾過に伴うすばやい消失のため，頻回投与による副作用の発現が問題となっていた．しかし，MTX-HSA複合体の開発に伴い，糸球体濾過の抑制に伴う半減期は，約15倍に上昇すると共に，浮腫部位への特異的蓄積が認められ，副作用の軽減にもつながることから，リウマチ様関節炎の新しい治療薬として臨床試験がスタートしている．

３）アルブミン融合技術によるコントロールドリリース

①生理活性ペプチドへの応用

米国ヒューマンゲノムサイエンス社はアルブミン融合技術をサイトカインに応用することに成功している．インターフェロン-HSA接合体；Albuferonは，血中におけるインターフェロンの貯蔵庫として機能するため，C型肝炎の治療に際し，副作用の軽減や投与回数の減少により，患者のQOL向上が期待されている[26]．さらに同社では，インターロイキンⅡにおいても同様にインターロイキンⅡ-HSA接合体；Albuleukinが開発されている．Albuleukinは，現在，第一相臨床試験が実施されている．Albuleukinの半減期は，インターロイキンⅡの約40倍長く，従来の投与設計を考えると，Albuleukinは，より簡易な投与設計を提供し，患者の負担軽減に貢献できる可能性を秘めている[27]．

②新規抗癌剤への応用

現在，新規抗癌剤として，血管新生阻害剤アンギオスタチンが注目されている．通常，固形癌は，その伸展・転移にあたり新生毛細血管を必要とするため，種々の血管新生促進因子を産生しているので，その抑制は癌を"兵糧攻め"することになる．また血管新生は正常細胞ではほとんど認められないので，血管新生阻害剤では重篤な副作用が起こりにくいと期待される．Bouquetらは，遺伝子融合技術を用いて，アンギオスタチンとHSAの接合体（Adk3-HSA）の開発を行い，Adk3-HSAの著明な半減期の延長と有意な腫瘍増殖阻害効果を得ている（Fig. 4.）[28]．

４）高機能性アルブミンの設計と評価

①ドメインⅠ，Ⅱ，Ⅲ

前述したようにHSAの基本構造は相同性の高い３つのドメインから構成されているが，蛋白質の機能解析を行う上でドメイン間の相互作用及び各ドメイン自身の機能解析は必要不可欠である．Carterらは，最近，各ドメインを単独に発現させる系を構築・精製し，相同性の高い３つのドメインの構造を明らかにすることにより，ドメイン毎の高い保存性を証明した．著者らは最近，各ドメインを単独に発現させる系を改良し，ドメイン

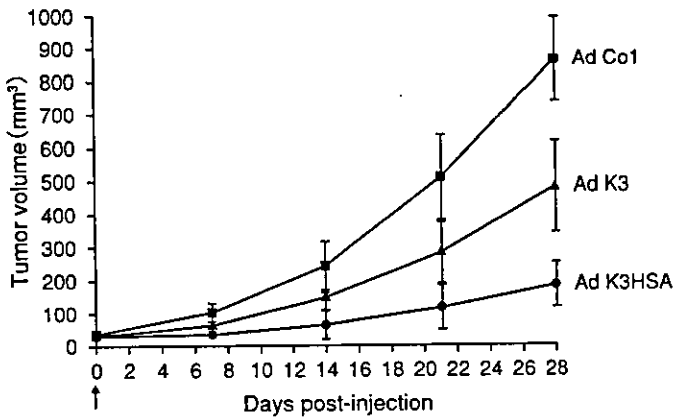

Fig. 4. Systemic treatment of MDA-MB-231 mammary tumors by AdK3-HSA. MDA-MB-231 carcinoma cells were sc implanted into athymic nude mice. 5×10^9 pfu of adenovirus AdK3-HSA (n = 12), AdK3 (n = 10), or AdCO1 (n = 8) was iv injected when the tumors had reached a mean volume of 30 ± 8 mm^3 (day 0). Data represent the tumor volume (mean ± SD) for each group. A Student t test was performed for statistical analysis ($P < 0.005$). (From Ref. 28)

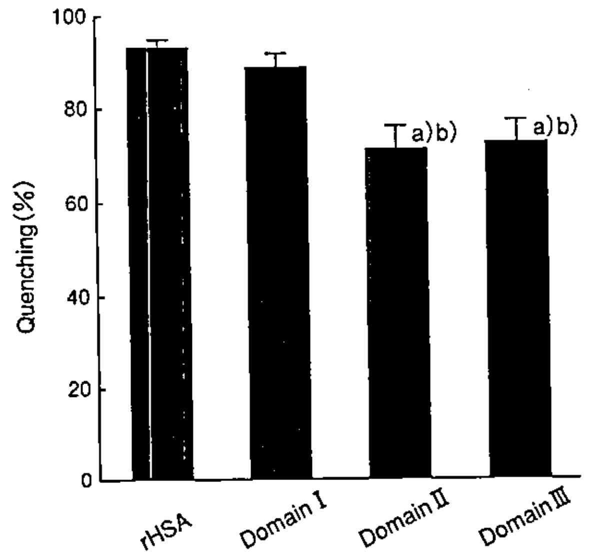

Fig. 5. Quenching of H_2O_2 oxidation of dihydrorhodamine (DRD) by rHSA and individual domains. The sample solutions contained 7.5 μM rHSA or individual domains in 67 mM sodium phosphate buffer (pH 7.4 and 25° C), 5 μM DRD, and 25 mM H_2O_2. Each bar represents the mean ± SD (n = 3). a) $p < 0.01$ vs. rHSA; b) $p < 0.01$ vs. domain I. (From Ref. 29)

毎の構造の安定化を立証するとともに，薬物結合サイトに関して，サイトⅠの構造及びミクロ環境保持には，局在しているドメインⅡだけでなく，隣接している他のドメインとの相互作用の重要性を明らかにした．また，サイトⅡは大部分がドメインⅢに位置し，ドメインⅢに保持されていることを明らかにした．加えて，ドメインⅠはドメインⅡ，Ⅲと比べ，抗酸化活性をよく保持していることが示された（Fig. 5.）[29]．前述したように，ドメインⅠの抗酸化能には34位のfreeのCys残基に加え，Met残基が影響を与えている可能性が考えられている．

②アルブミンダイマー（$rHSA_2$）

従来，アルブミンのダイマー化では，架橋剤を利用した化学的結合のため，均一な結合体の調製の難しさなど多くの問題を抱えていた．最近，W.P.Sheffieldらにより，遺伝子技術を用いた均一なラビット二量体化アルブミンが精製された．しかし，その半減期は，予想に反しwild-typeに比べ短縮していた[30]．この原因として，均一な二量体は発現しているものの，発現・精製過程におけるアルブミン自身の構造変化が惹起され，消失を促進したものと推察される．事実，著者らも同様に二量体化HSAの発現・精製を行った結果，構造及び機能特性においてHSA-monomerと同様であることを確認した．またその体内動態は，HSA-monomerに比べ，有意に延長していた．これらの知見から，二量体化HSAの生体内挙動は，分子サイズに加えて，その構造安定性により大きく左右されるかもしれない．

5）高機能性アルブミンを利用した血液透析への応用

HSAの高い結合率を利用した医薬への応用の一つにアルブミン透析（ECAD; Extra Corporeal Albumin Dialysis）がある．肝不全は種々の毒素の体内蓄積により他の臓器を障害し，しばしば多臓器不全に進展する．治療として血液濾過透析などの人工肝機能補助療法が行われているが，アルブミンに結合した毒素は除去できず血漿交換との併用が必要である．そこで，ECAD療法により，水溶性毒素とともにアルブミン結合毒素を選択的に除去するシステムである．6時間の治療で血清胆汁酸，ビリルビンが有意に低下することに加え，血漿Nox濃度も有意に低下することから，毒素の除去に伴う血管系，脳，腎及び肝機能の改善に伴う余命の延長が報告されている（Fig. 6.）[31,32]．

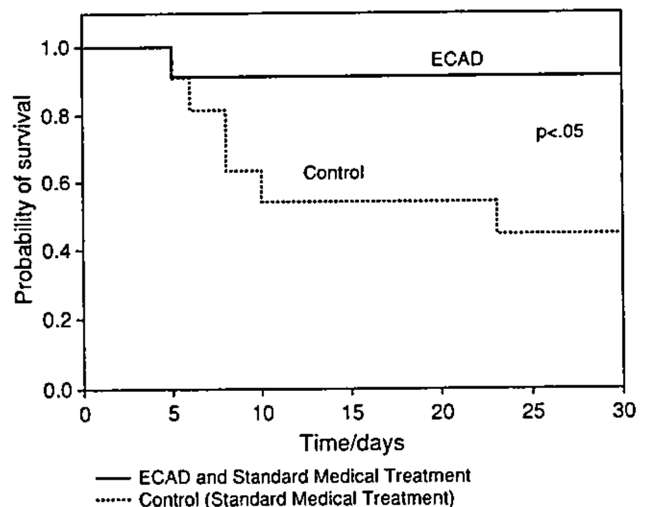

Fig. 6. Kaplan-Meier plot showing 30-day mortality. There was a significant difference between the extracorporeal albumin dialysis and control group ($P < .05$). (From Ref. 32)

このECAD療法に際し，遺伝子組換え型アルブミン，特に高結合能アルブミンの開発は，毒素を結合させるECAD療法において，有用な変異体として利用されうるかもしれない．

おわりに

遺伝子組換え技術により，作製されたHSA変異体により機能発現に必須なアミノ酸残基及びその発現部位が明らかにされてきた．また，機能によって，その発現部位の範囲やドメイン間相互作用の関与に大きな違いが認められた．今後，遺伝子組換え技術により，各ドメインを組み合わせたり，HSAの単量体や多量体分子と接合させることにより，医薬への応用を目指した高機能性組換え型HSAの設計が可能になるかもしれない．

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Bucolome, a potent binding inhibitor for furosemide, alters the pharmacokinetics and diuretic effect of furosemide: potential for use of bucolome to restore diuretic response in nephrotic syndrome

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Abstract

To determine whether bucolome (5 - n - butyl - 1 - cyclohexyl - 2, 4, 6 - trioxoperhydropyrimidine), a nonsteroidal anti-inflammatory agent, can reverse diuretic resistance of furosemide in patients with nephrotic syndrome, we examined the inhibitory effect of bucolome on the protein binding of furosemide in serum and urine. Bucolome significantly inhibited the protein binding of furosemide not only in serum but also in urine of preparation albumin (UPA), which mimics urinary albumin concentration in patients with nephrotic syndrome by ultrafiltration method. The binding percentage of furosemide to albumin was approximately 70 % in UPA. With coadministration of bucolome to healthy volunteers, renal clearance of furosemide was increased, reflecting the increase of the free fraction of furosemide in serum. Furthermore, coadministration of bucolome caused a significant increase of urine volume and sodium concentration in urine. Even at higher urine levels of furosemide, the inhibitory effect of bucolome on the protein binding of furosemide in UPA remains constant, and changes in pH at weakly acidic pH levels (pH 5.5-6.5) did not alter the inhibitory effect of bucolome. Interestingly, coadministration of bucolome with furosemide in adriamycin-induced nephrotic syndrome model rats alleviated the diuretic resistance. These results suggest that bucolome has a potent inhibitory effect on the protein binding of furosemide in the urine, and can partially restore the diuretic response of furosemide in patients with nephrotic syndrome by increasing the free fraction of furosemide at the site of action.

Introduction

Attenuated response to diuretics is frequently observed in patients with nephrotic syndrome (Green and Mirkin, 1980; Keller et al., 1982; Smith et al., 1985; Kirchner et al., 1990; Kirchner et al., 1992; Wilcox, 2002). Dose-response analysis comparing urinary excretion rates of diuretic and sodium has demonstrated that nephrotic patients are less responsive to a given dose of drug than healthy subjects (Smith et al., 1985). It has been proposed that this blunted response is due to tubular resistance to the natriuretic effect of loop diuretics. Another possible contribution to diuretic resistance in patients with nephrotic syndrome is suggested by the finding that furosemide readily binds to albumin in serum (Takamura et al., 1996; Takamura et al., 1997; Takamura et al., 1998), which implies that it also binds to albumin in urine. As the protein bound fraction of a drug is considered to be inactive (Martin, 1965; Meyer and Guttman, 1968; Vallner, 1977), binding of furosemide to urinary albumin would decrease the effective drug concentration by reducing its unbound fraction at its site of action in the thick ascending limb of Henle's loop. This suggests that binding inhibitors for furosemide can normalize the natriuretic effect of the drug in renal tubules.

To have a significant renal effect, a protectable inhibitor should have the following properties: 1) it should be a potent inhibitor of the protein binding of furosemide in urine; 2) when administered in large doses, its plasma concentration should reach high levels; 3) it should be excreted mainly in urine; and 4) it should be highly safe and suitable for repeated administration.

Kirchner *et al.* reported that binding inhibitors such as warfarin and sulfisoxazole alleviated the blunted response to furosemide in rats in their *in vivo* loop microperfusion experiments (Kirchner et al., 1991). However, warfarin is eliminated by metabolism rather

than urinary excretion, despite it has high affinity for albumin (Kragh-Hansen, 1981; Maes et al., 1982). In addition, warfarin can only be administered in small doses. Some sulfa drugs including sulfisoxazole and sulfamethizole are eliminated mainly by urinary excretion but have low affinity for albumin (binding affinity, nK = $1 \times 10^4 M^{-1}$ to $5 \times 10^4 M^{-1}$) (Vallner, 1977; Kragh-Hansen, 1981), suggesting that these drugs would not be effective in restoring the blunted response to furosemide in patients with nephrotic syndrome (Agarwal et al., 2000).

Previously, we reported that furosemide binds to site I on human serum albumin (HSA), which is one of the major binding sites for drugs on HSA. Also, we found that valproic acid, phenytoin and bucolome inhibited binding of furosemide to HSA (Takamura et al., 1996). Bucolome, a nonsteroidal anti-inflammatory drug, can be administered in large doses (600 to 1200 mg/day); its plasma concentration reaches about 300 μM; and it is excreted mainly in urine (Kakemi et al., 1970; Yashiki et al., 1971a; Yashiki et al., 1971b; Chiba et al., 1985).

In the present study, we investigated the effects of bucolome on the pharmacokinetics and pharmacodynamics of furosemide in healthy subjects and adriamycin-induced nephrotic syndrome model rats. First, we studied the effects of bucolome on protein binding of furosemide in serum and urine, and found that bucolome is an effective binding inhibitor for furosemide. Moreover, we found that coadministration of bucolome affects the pharmacokinetics of furosemide, based on the inhibition of protein binding, suggesting an increased natriuretic effect of the diuretic. These results suggest that bucolome can alleviate diuretic resistance in patients with nephrotic syndrome.

Materials and Methods

Materials

Furosemide powder and furosemide intravenous solution (20 mg/mL) were donated by Hoechst Japan Co. (Tokyo, Japan). Adriamycin was donated by Kyowa Hakko Kogyo Co. (Tokyo, Japan). HSA (essentially fatty acid free) and rat serum albumin (RSA) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). RSA was defatted with activated charcoal in solution at 0 °C, acidified with H_2SO_4 to pH 3, and then freeze-dried (Tsutsumi et al., 1999). Bucolome (5-n-butyl-1-cyclohexyl-2,4,6-trioxoperhydropyrimidine, Grelan Pharmaceutical Co. Ltd., Tokyo, Japan) was obtained as a pure substance from the manufacturer. All other chemicals were of analytical grade.

Methods

Protein Binding Study

Ultrafiltration experiments were performed using a Tosoh plastic ultrafiltration apparatus (Kanagawa, Japan). Aliquots (0.9 or 1.35 mL) of different concentrations of furosemide and a constant concentration of HSA (in 63 mM sodium phosphate buffer, pH 7.4), serum protein, RSA or urine of preparation albumin (UPA), which mimics urinary albumin concentration observed in patients with nephrotic syndrome by adding the HSA to urine of healthy volunteers, were centrifuged at 1200 or 2200 g for 15 min at 25 °C. Adsorption of drugs onto the membrane or apparatus was negligible. No protein leakage was detected during the experiment. .

The free concentrations of furosemide and bucolome were determined by HPLC using a system consisting of a Shimadzu LC-6A UV detector and a Shimadzu SPD-6A pump. For both drugs, a Superspher 100 RP-18e column (Cica Merck, Tokyo, Japan) was used as the

stationary phase. Both furosemide (Retention time, 4.3 min) and bucolome (Retention time, 5.3 min) were assayed by UV monitoring at 280 nm. HPLC was performed with a flow rate of 1.0 mL/min at 40 °C with a column oven, using distilled water / acetonitrile / methanol (491 : 9 : 4 v/v/v) and distilled water / acetonitrile (119:6 v/v) as the mobile phase for furosemide and bucolome, respectively.

Effect of Bucolome on the Natriuretic Effect of Furosemide in Healthy Volunteers

The protocol was approved in advance by the Ethical Committee of Miyazaki College Hospital. A member of the volunteer was asked to give informed consent when they were unable to do so, according to Japanese legislation. The subjects were 3 normal male humans, 34 to 48 years old and weighing 65 to 79 kg, who participated as outpatients. Each subject had a normal medical history, physical condition, and standard laboratory test results. Sodium and fluid intake were maintained at constant levels in all experiments. It has been reported that t_{max} of bucolome is 4.5 hours in man (Yashiki et al., 1971b). In order to obtain the maximum displacement effect by bucolome for the protein biding of furosemide, each subject was intravenously administered 20 mg of furosemide alone or 4.5 hours after pretreatment with bucolome (600 mg, p.o.). An interval of at least 2 weeks elapsed between experiments (crossover fashion). Blood samples (6 mL) used to determine drug concentrations were obtained 5, 10, 15, 30, 60, 120 and 240 min after administration of furosemide. The blood was immediately centrifuged at 3000 rpm for 10 min, and the serum was stored at -80 °C until analysis for furosemide. In separate experiments, free furosemide concentration in humans was determined by ultrafiltration techniques.

The concentration of furosemide in serum was determined using the HPLC system described above. An Inertsil ODS column (5 μm) (GL Sciences Inc., Tokyo, Japan) was used as the stationary phase. A 0.2-μg aliquot containing the internal standard, ethyl *p*-

hydroxybenzoate, was added to 0.1-mL serum samples. This mixture was vortexed, followed by addition of 0.5 mL of 1 M potassium dihydrogenphosphate and 3 mL of diethylether. The mixture was shaken for 10 min and then centrifuged (3000 rpm) for 10 min. The upper layer (2 mL) was transferred to a clean test tube and evaporated under nitrogen to dryness. Then, 250 µL of mobile phase was added to the tube, and the resultant mixture was injected into the HPLC apparatus. HPLC was performed at a flow rate of 1.1 mL/min at 40°C with a column oven, using 50 mM potassium dihydrogenphosphate / acetonitrile (68 : 32 v/v) as the mobile phase for furosemide (Retention time, 8.0 min). An UV monitor was used to assay for furosemide and ethyl *p*-hydroxybenzoate, at a UV wavelength of 270 nm for both.

Analysis of *In Vitro* Binding of Furosemide

Binding parameters were estimated by fitting the experimental data to the following equation using a nonlinear least-squares computer program (MULTI) (Yamaoka et al., 1981):

$$r = \frac{[D_b]}{[P_t]} = \sum_{i=1}^{j} \frac{n_i K_i [D_f]}{1 + K_i [D_f]} \qquad (1)$$

where r is the number of moles of bound drug per protein molecule; $[D_b]$ and $[D_f]$ are the bound and unbound drug concentrations; $[P_t]$ is the total protein concentration; and K_i and n_i are the binding constant and the number of binding sites for the class of binding sites. The simultaneous binding of two ligands was analyzed using a previously reported method (Kragh-Hansen, 1981), as follows:

$$r_A = \frac{[A_b]}{[P_t]} = \frac{K_A[A_f] + \chi K_{BA} K_B [A_f][B_f]}{1 + K_A[A_f] + K_B[B_f] + \chi K_{BA} K_B [A_f][B_f]} \qquad (2)$$

$$r_B = \frac{[B_b]}{[P_t]} = \frac{K_B[B_f] + \chi K_{AB} K_A [A_f][B_f]}{1 + K_A[A_f] + K_B[B_f] + \chi K_{BA} K_B [A_f][B_f]} \qquad (3)$$

where K_A and K_B are the binding constants of ligands A and B, $[A_f]$ and $[B_f]$ are the free concentrations of ligands A and B, and $[A_b]$ and $[B_b]$ are the bound concentrations of ligands A and B. χ is a coupling constant, K_{BA} is the binding constant of ligand A in the presence of ligand B, and K_{AB} is the binding constant of ligand B in the presence of ligand A. Using these equations, we calculated the theoretical values of χ. The interaction mode of the ligands on a macromolecule can be evaluated from the sign and magnitude of the value of χ. For example, if ligands A and B are independently bound to protein, χ is equal to 1. The results $\chi > 1$ and $0 < \chi < 1$ indicate cooperative and anti-cooperative interaction between ligands. Competitive displacement between ligands is indicated by $\chi = 0$. In this analysis, $r < 0.42$ was used to suppress the contribution of low-affinity binding site.

Inhibition of furosemide binding by bucolome was estimated from monitored changes of the free ligand fraction, f_u, which was calculated as follows:

$$f_u = \frac{[D_f]}{[D_f]+[D_b]} \qquad (4)$$

Pharmacokinetics of Furosemide in Normal and Nephrotic Syndrome Model Rats

Adult male Wistar rats were housed in an air-conditioned room with free access to commercial feed and water, and fasted for 16 h before the abdominal aorta injection. All animal experiments were conducted according to the guideline principle and procedures of Kumamoto University for the care and use of laboratory animals. Experimental nephrotic syndrome was induced in rats by administration of adriamycin in a single injection (7.5 mg/kg) via the tail vein (Nephrotic Syndrome model rats, NS rats) (Bertani et al., 1982). Control rats were injected with isotonic saline. Male Wistar rats (250-290 g; 2 weeks after i.v. administration of adriamycin or saline) underwent the surgical procedure under light anesthesia with diethyl ether. A cannula was inserted into the femoral vein and artery, using

polyethylene tubing (polyethylene-50; i.d., 0.58 mm; o.d., 0.9655 mm; BD Biosciences, Parsippany, NJ). The bladder was also cannulated with polyethylene tubing (polyethylene-8; o.d., 2.33 mm; Hibiki Co., Tokyo, Japan.). The body temperature of the rats was maintained by heating with a lamp. Thirty minutes before the i.v. injection of furosemide, control samples of urine were collected. Furosemide was administered at a dose of 2 mg/kg by rapid infusion into the femoral vein, alone or 1 hour after oral pretreatment with bucolome as well as human study (t_{max} of bucolome in rats = 53.5 min, data not shown). After infusion, the cannulae were flushed with a small volume of heparinized saline, to ensure that the complete dose was administered, and to prevent the formation of clots. Blood samples (200 μL) and urine were taken from the femoral artery and bladder, respectively, at designated times. The blood was placed in graduated microcentrifuge tubes (0.6 mL) that contained a drop of heparinized saline as an anticoagulant. The blood samples were centrifuged (1500 *g* for 10 min), and the plasma was removed.

Plasma concentration profiles were analyzed by fitting the following biexponential equation with the nonlinear least-squares method (MULTI):

$$C_p = A \cdot \exp(-\alpha \cdot t) + B \cdot \exp(-\beta \cdot t) \qquad (5)$$

Pharmacokinetic parameters were calculated using the following equations:

$$AUC_{0\rightarrow\infty} = A/\alpha + B/\beta \qquad (6)$$

$$CL_{tot} = Dose/AUC \qquad (7)$$

$$t_{1/2\beta} = 0.693/\beta \qquad (8)$$

where $AUC_{0\rightarrow\infty}$, CL_{tot}, and $t_{1/2\beta}$ represent AUC from zero to infinity, total body clearance and half-life of the β phase, respectively.

Statistical Analysis

Student's *t* test was used to analyze differences between two groups. ANOVA was used to analyze differences among more than two groups, and the significance of difference between two means in these groups was evaluated using the modified Fisher's least squares difference method.

Results

Effect of Bucolome on the Protein Binding of Furosemide *In Vitro*

In order to estimate the effect of bucolome on the protein binding of furosemide, we have measured the free fraction of furosemide in human serum in the absence and presence of bucolome. Protein binding of furosemide (20 μM) was significantly inhibited by 300 μM of bucolome, and f_u of furosemide in the presence of bucolome (f_u = 3.00 ± 0.12 %) was approximately 3-fold greater than the value in the absence of bucolome (f_u = 1.10 ± 0.09 %).

Figure 1 shows the results of a quantitative analysis of mutual displacement between furosemide and bucolome. The kinetic binding constant ($n_1 \cdot K_1$) of furosemide on the binding to HSA was reported to be 2.0 x 10^5 M^{-1}(Takamura et al., 1996), and that of bucolome was determined to be 1.5 x 10^6 M^{-1} (data not shown). Taking their parameters into consideration, 60 μM of these ligands will inhibit the binding to albumin. In this condition, the binding isotherm of furosemide in the presence of bucolome, and vice versa, was fairly close to the theoretical curve that assumed competition between these two ligands at a common site.

Effect of Bucolome on the Pharmacokinetics of Furosemide in Healthy Volunteers

Table I summarizes the pharmacokinetic parameters of furosemide after intravenous administration (20 mg) with and without oral coadministration of bucolome (600 mg) in healthy volunteers. In the group with coadministration of bucolome, AUC decreased significantly, and CL_{tot}, renal clearance (CL_r) and steady-state volume of distribution (V_{dss}) increased significantly. However, $t_{1/2\beta}$ was unchanged. These results suggest that the alteration of pharmacokinetics of furosemide was caused by inhibition of protein binding by bucolome. In fact, at 5, 10 and 15 min after administration of furosemide, the free fraction of furosemide increased significantly in the group with coadministration of bucolome (Fig. 2).

Effect of Bucolome on the Natriuretic Effect of Furosemide in Healthy Volunteers

In order to evaluate the pharmacodynamics of furosemide, urine volume and sodium concentration in urine were measured at 6 hours after intravenous administration of furosemide. As mentioned above, in the group with coadministration of bucolome, renal clearance of furosemide was increased (Table I). This result is consistent with the significant increase of urine volume and sodium concentration in urine (Table II).

Effect of Bucolome on the Protein Binding of Furosemide in Urine

To restore the diuretic response in patients with nephrotic syndrome, a binding inhibitor (bucolome) must displace the diuretic (furosemide) in urine containing a high concentration of albumin. To determine whether bucolome inhibits the protein binding of furosemide in urine, ultrafiltration was performed using artificial nephrotic urine, which contains a level of albumin equivalent to that of patients with nephrotic syndrome. The results indicate that bucolome inhibited the protein binding of furosemide in the artificial nephrotic urine (Fig. 3A). This inhibition was not affected by changes in the pH of urine that simulated the acidosis that occurs in nephrotic syndrome (Fig. 3B).

Effect of Bucolome on the Pharmacokinetics of Furosemide in Normal Rats

Table III summarizes the pharmacokinetic parameters of furosemide after intravenous administration (2 mg/kg) with and without oral coadministration of bucolome in normal rats. An increase in the dose of bucolome caused a significant decrease of AUC, and a significant increase of CL_{tot}, CL_r, V_{dss} and f_u.

To confirm whether furosemide and bucolome share the same binding site on RSA, we studied the interaction between furosemide and bucolome. *In vitro* binding analysis indicated that bucolome inhibited the binding of furosemide competitively (data not shown). Furosemide and bucolome have approximately the same affinity for RSA (furosemide, $K_a = 4.30 \times 10^6 \pm 0.64 \times 10^6\ M^{-1}$; bucolome, $K_a = 6.63 \times 10^6 \pm 1.79 \times 10^6\ M^{-1}$). The affinity of furosemide for RSA was greater than its affinity for HSA (primary binding affinity $K_1 = 2 \times 10^5\ M^{-1}$(Takamura et al., 1996)), whereas bucolome had approximately equal affinity for RSA and HSA ($K_1 = 1.5 \times 10^6\ M^{-1}$). The strong agreement between these findings and the pharmacokinetics and binding mode of furosemide in human and rats suggests that the present NS rats constitute a relevant model for analysis of interaction between furosemide and bucolome.

Effect of Bucolome on the Diuretic Effect of Furosemide in Normal and NS rats

Adriamycin-treated rats exhibited symptoms of nephrotic syndrome. In NS rats, serum albumin was significantly decreased, and urinary albumin was markedly increased (Table IV). Body weight was significantly decreased in NS rats, and hematocrit and urine volume tended to be lower in NS rats than in normal rats.

In order to evaluate the effect of bucolome on diuretic resistance in NS rats, urine volume and furosemide excretion into urine were measured. Urine volume increased with increasing dose of bucolome, which is consistent with the significant increase of urinary

excretion of furosemide in normal rats (Fig. 4, A and C). Although NS rats exhibited attenuation of diuretic response, compared with normal rats (Fig. 4, C and D), diuretic resistance was restored by oral coadministration of bucolome, accompanied by an increase of urinary excretion of furosemide (Fig. 4, B and D).