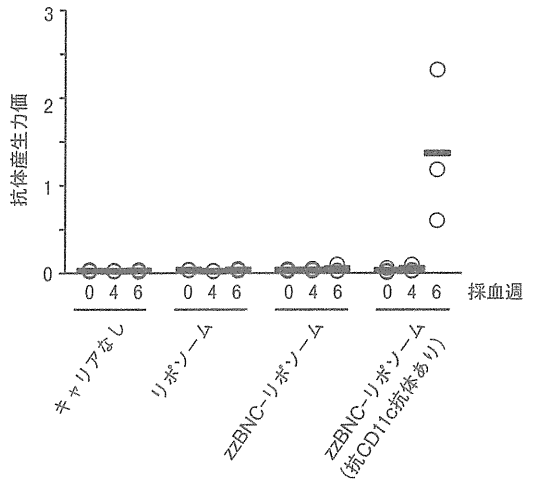

○；個々のマウスにおける抗体産生力価，$n = 3$，太線；抗体産生力価平均値。

図7　バイオナノカプセルによる攻めのワクチン

抗CD11c抗体をzzBNCに提示し，日本脳炎ウイルス抗原を内包したリポソームと複合体を形成させてマウスに免疫し，各時間で採血して抗体産生を検定した。

zzBNCは少量の抗体でも十分な標的化能を発揮できる理想的な機能を有している。

6. バイオナノカプセルの展開

BNCのモデルであるHBVは，血液中に侵入して肝細胞に感染しHBVの複製を行うまでに，実に多くの生体防御壁をすり抜ける。その機構は実に複雑かつ巧妙であり，血中滞留時には，①RES回避能，②免疫系回避能，③標的組織・細胞標的化能，④細胞膜融合能，細胞内へと侵入した後では，⑤エンドソーム脱出能，⑥核内移行能，⑦転写活性化能，と全ての能力をフル活用する。最近，われわれはBNCの細胞内侵入能はHBVに匹敵することを明らかにしており[33]，前述の知見と併せてBNCには上記HBVの有する機能の①から⑤が搭載されていると考えている。一方，これまでのDDSキャリアは，生体レベルの動態解析に基づきマクロレベルでの最適化が進められてきたが，細胞内および生体内解析技術の進展により，送達する薬剤の種類に従って，細胞外，細胞膜，細胞質，エンドソーム，核へとミクロレベルで細胞内動態を最適化する必要性が高まっている。BNCについても，今後BNC製剤を開発するに当たっては，当然細胞内動態を解析する必要がある。特に，前記機能③および④の発現に際して，HBVが肝細胞表面のヘパラン硫酸プロテオグリカンなどのHBV低親和性受容体と結合し，次にHBV高親和性受容体に引き渡されて，その後細胞内に侵入すると考えているが[33]，今なおHBV高親和性受容体は未同定であり，BNCの細胞内動態を解析するには，まず本受容体解析を行うことが急務である。次に，前記機能⑤の発現に際して，pre-S1領域N末端20アミノ酸残基に存在する膜融合活性領域が低pH依存的に活性化すると考えられているが，その詳細な作用機構については明らかになっていない。さらに，遺伝子送達においては，核内へと治療遺伝子を送達させる必要があるが，核膜のポアを通過するにはATP依存的な能動輸送タンパク質が関与しており，核酸のような巨大分子の通過は困難である。したがって，BNCを用いる核酸送達においては，HBVにおけるコアタンパク質のような能動的核内移行メカニズムを付与する必要がある。以上のように，BNCには解明すべき現象，搭載すべき機能が多く残されている。

【謝　辞】

本研究は，㈱農業・食品産業技術総合研究機構・生物系特定産業技術研究支援センター・イノベーション創出基礎的研究推進事業（技術シーズ開発型・一般枠），日本学術振興会科学研究費補助金（基盤研究A，21240052），厚生労働科学研究費補助金（B型肝炎創薬実用化等研究事業）の支援による成果であり，ここに謝意を表します。

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バイオナノカプセルによる生体内ピンポイント薬物・遺伝子送達技術

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ウイルスは，生体内の様々な異物排除系を巧妙にすり抜け，組織・細胞に特異的に感染し，自己のゲノム情報を宿主に伝播するナノ構造体である。このウイルスからゲノムを取り除き，代わりに薬剤や治療遺伝子を封入した中空ナノ粒子は，生体内の細胞を能動的に標的化でき，かつ安全で生産性の高い次世代のナノ医療技術を支えるDDS（drug delivery system）キャリアになる可能性がある。本稿では，ヒトB型肝炎ウイルス（HBV：hepatitis B virus）から着想を得た非ウイルス性DDSキャリアであるバイオナノカプセル（BNC：bionanocapsule）の開発と応用展開について紹介する。

1. はじめに

様々な疾患に対する医薬品（低分子化合物，ペプチド，タンパク質，DNA，RNA）の開発が進む中，薬剤の生体内安定性を高めたり，患部以外での作用（広義の副作用）を低減するため，"必要な時に，適切な分量の薬剤を患部に徐放（controlled release）させる技術"の開発も併せて重要視されてきている。この薬剤送達システム（DDS）を成立させるものとして，患部に指向性を示す種々のナノスケールのDDSキャリアが開発されている。現行のDDSキャリアは全般的に，直径50~150 nmのナノ粒子であり，薬剤（化合物や核酸等）と複合体を形成できるようデザインされている。例えばリポソーム（脂質二重層のカプセル）は，内部に薬剤を封入でき，血管新生の盛んな増殖性固形癌へEPR（enhanced permeation and retention）効果[1]により集積する受動的標的型DDSキャリアとして用いられる。しかし，そのままでは血液中の補体成分による分解や，肝臓や脾臓に存在する細網内皮系（RES：reticuloendothelial system）[2]での貪食が起きるので，リポソーム表面をPEG（polyethylene glycol）で修飾して，生体内での安定性を狙ったステルス化が行われている[3]。ミセルもステルス化を担うPEGなどから構成される親水部と，アルキル鎖などから構成される疎水部を有し，会合することによって外部に親水部を露出しつつ内部に疎水性部を形成し，疎水性部にシスプラチンなどの難溶性薬剤を包含可能にしている[4]。リポソームやミセルに基づくDDSキャリアは，世界各国で臨床応用されているが，投与初期に誘導される抗PEG IgM抗体により，以降の投与において血中からのクリアランスが異常に早くなる現象（ABC（accelerated blood clearance）効果）[5,6]も報告されており，頻回投与が困難となる場合もある。核酸医を送達可能とするポリマーでは，例えばPEI（polyethyleneimine）のように強い正電荷を有することが多く，静電的相互作用により種々の核酸（治療遺伝子やsiRNA等）と複合体を形成することができる。しかし，ポリマーは自己組織化されにくい性質を有することから性状（電荷や粒子径）の制御が難しく，また強い表面電荷をもつことからRESにより捕捉され易い欠点がある。非標的組織への誤送達や正常組織に対する毒性も問題になっている[7]。ウイルスの中には，RESを回避しつつ標的組織を細胞レベルで特異的に認識して自己のゲノム情報を宿主に導入するものがあり，この性質を利用して癌組織や癌以外の疾患組織へも薬剤を送達できる能動的標的型GDS（gene delivery system）

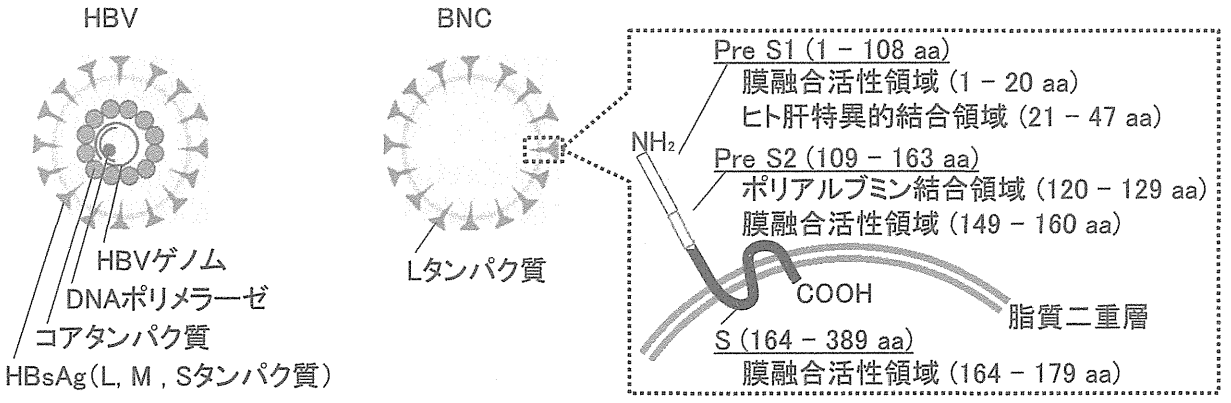

Fig. 1 HBV と BNC

キャリアとして用いる試みがある。しかし現状，遺伝子治療で臨床応用されているウイルスベクター（アデノウイルス，アデノ随伴ウイルス，センダイウイルス，レトロウイルス等）[8]は，遺伝子導入効率の高さに反して組織・細胞特異性を示すものは極めて少ない。これは患部以外へのウイルスゲノムの偶発的な導入リスクの原因となり，事実，欧米では死亡事故も発生している[9,10]。このように，現行の DDS には一長一短があり，本稿では，以上の問題を解決する DDS キャリア BNC とその応用展開について紹介する。

2. HBV から着想を得た BNC

我々が開発する BNC は，極微量のコピー数でもヒト肝臓に特異的に感染して肝炎（肝癌まで）を惹き起こす HBV を模したウイルスゲノムフリーの中空ナノ粒子である。HBV のエンベロープに埋め込まれた表面抗原タンパク質（HBsAg：HBV surface antigen）は，pre-S1 領域（108 aa），pre-S2 領域（55 aa），および S 領域（227 aa）からなる L タンパク質，pre-S2 および S 領域からなる M タンパク質，S 領域のみからなる S タンパク質の 3 つから構成される（Fig.1）[11]。肝実質細胞への特異性は L タンパク質の pre-S1 領域の N 末端側 21-47 アミノ酸残基にあり[12]，細胞表面の受容体への結合を介して同領域（特に我々が新規に同定した N 末端側 20 aa，投稿中），pre-S2 の C 末端側，および S 領域の N 末端側に存在する膜融合活性を利用して標的細胞内に侵入する[13,14]。さらに Pre-S2 領域は，ポリアルブミンと結合する部位（PAR：poly-albumin receptor）を有し[15]，血液中でアルブミン重合体と結合し，血中タンパク質との結合（オプソニン化）を抑制して RES を回避する役割を担うと考えている（投稿中）。さらに HBV の中には B 型肝炎ワクチンを接種した者でも感染発症する免疫エスケープ変異体（HBsAg 遺伝子配列から共通する 2 箇所のアミノ酸変異）が存在することから，DDS キャリアとしての課題である低免疫原性も達成される可能性が高い。このように，HBV は理想の DDS キャリアに必要な原型（細胞特異性，RES 回避能，低免疫原性）を有しており，我々は HBsAg をリポソームに埋め込むことにより HBV 由来の能動的標的化能と RES 回避能をもつ低免疫原性のウイルスゲノムフリーの中空ナノ粒子 BNC を創成した（投稿中）。

3. BNC の性状と大量生産系

ヒト肝臓特異的結合領域，膜融合活性領域（pre-S1，pre-S2，S の 3 箇所に存在），PAR を有する HBsAg・L タンパク質を出芽酵母で発現して得られるウイルスゲノムフリーの BNC を用いる。BNC は直径約 80 nm の中空ナノ粒子で，内部に遺伝子や薬剤を封入することができる（Fig.1）[16–19]。BNC は遺伝子組み換え出芽酵母により発現・精製され，酵母の全可溶性タンパク質の 40%以上を占め，培養液 5L から精製 BNC を再現性良く 10 mg 以上取得できる。また，酵母ゲノムや酵母由来タンパク質のコンタミネーションは検出限界以下であることを定量的 PCR 法や ELISA により確認しており，GMP（good manufacturing protocol）準拠の医薬品として大量生産が可能である。事実，出芽酵母を用いる本 BNC 生産法は既に承認されている B 型肝炎ワクチンと同様である。出芽酵母内で発現された L タンパク質は，小胞体膜上で S 領域間の分子間認識により自己組織化し，小胞体ルーメン側に BNC として放出される。BNC 1 粒子には約 114 個の L タン

パク質が酵母小胞体膜由来リポソーム上に膜タンパク質として組み込まれ，pre-S1 及び pre-S2 領域を外側に提示して存在する。BNC は非常に安定であり，70 °C で 1 時間加温しても機能は損なわれず，凍結乾燥することにより 4 °C で 14ヶ月以上保管できることを確認している。凍結乾燥物は滅菌水を添加することにより容易に水和し，元の性状へと復帰できる。

4. BNC への薬剤封入法と薬剤送達

BNC 内部への薬物封入は当初，電気穿孔法により行った（Fig.2A）。GFP（green fluorescence protein）発現遺伝子や蛍光化合物カルセインなどを本法により BNC 内に導入し，*in vitro* で標的細胞に作用させると，市販のリポソーム法を原理とする一般的 DNA 導入試薬と比べて，単位 DNA 当たり 100 倍以上の遺伝子導入効率を示した〔19〕。また，ヒト肝癌細胞由来腫瘍を有する担癌マウスに同 BNC を尾静脈注射すると，*in vivo* で同腫瘍に特異的に薬剤送達することに成功した。この時，コントロールとして作製したヒト大腸癌細胞由来腫瘍には送達されず，またマウスの主要臓器への BNC の移行は認められなかった。

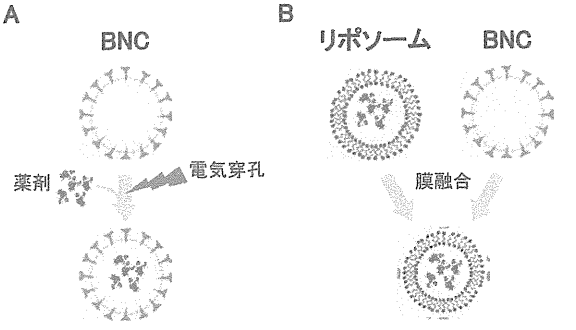

Fig. 2 BNC への薬剤封入法

このように，BNC は正確な組織・細胞特異性と，高い薬剤・遺伝子導入効率を示したが，電気穿孔法による薬剤封入は創薬の観点から大量生産に不向きである。そこで，BNC の膜融合活性を利用し，BNC-リポソーム複合体を形成させる方法を考案した（Fig.2B）。BNC は遺伝子を包含したカチオン性リポソームや薬剤を包含したアニオン性リポソームと複合体を形成し，直径 100~150 nm 程度のナノ粒子として取得され，大量調製が可能になった〔20〕。BNC-リポソーム複合体は BNC の対リポソーム重量比が 5%程度でも *in vitro* において細胞特異性を示し，遺伝子包含 BNC-リポソーム複合体はリポソーム単独に比べて飛躍的な遺伝子導入効率を示した。また，抗癌剤ドキソルビシン（Dox）包含 BNC-リポソーム複合体は，ヒト肝癌細胞由来腫瘍を有する担癌マウスにおいて，4 mg/kg の薬剤量で Dox 単体や Dox 包含リポソームよりも高い抗腫瘍活性を示した〔21〕。また，Dox 包含 BNC-リポソーム複合体は，市販の Dox 内包 PEG 化リポソーム製剤 Doxil よりも腫瘍内において高濃度を長時間維持し，血中滞留性も Doxil に匹敵した。

一方 BNC は，リポソームだけでなくポリマーとも複合体を形成可能なことを確認している。現状，ポリマーは主に受動的標的型 GDS キャリアとして用いられているが，代表的な PEI の場合，生体内への単体投与では肺に集積する一方，細胞膜状に普遍的に存在するプロテオグリカンと非特異的に結合するため，非特異的送達の回避は困難であった。我々は，BNC とルシフェラーゼ遺伝子と複合体を形成した PEI を混合し，直径約 200 nm の BNC-PEI-DNA 複合体を作製したところ，*in vitro* でヒト肝癌由来細胞特異的に対し高い遺伝子導入活性を示すことを見出した（Fig.3）〔22〕。

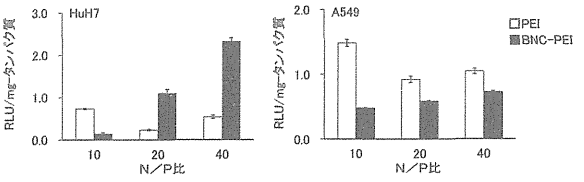

Fig. 3 BNC-PEI 複合体を用いたヒト肝臓細胞特異的送達
N/P 比は PEI の正電荷に寄与する窒素原子数/DNA の負電荷に寄与するリン原子数を表す。RLU, relative luminescence units; HuH7, 肝癌細胞株; A549, 肺腺上皮癌細胞株。

また，同複合体をヒト肺癌細胞由来組織およびヒト肝癌細胞由来組織の両方を有する担癌マウスに静脈注射したところ，ヒト肝癌細胞由来組織に特異的に集積していた（Fig.4）。この結果は，BNC が PEI に HBV に由来する肝標的化能を付与し，同時に PEI 特有の肺への集積を抑制したことを意味する。

5. BNC の再標的化

ここまで BNC は，ヒト肝臓特異的な DDS キャリアとして有用であることを紹介したが，我々の研究

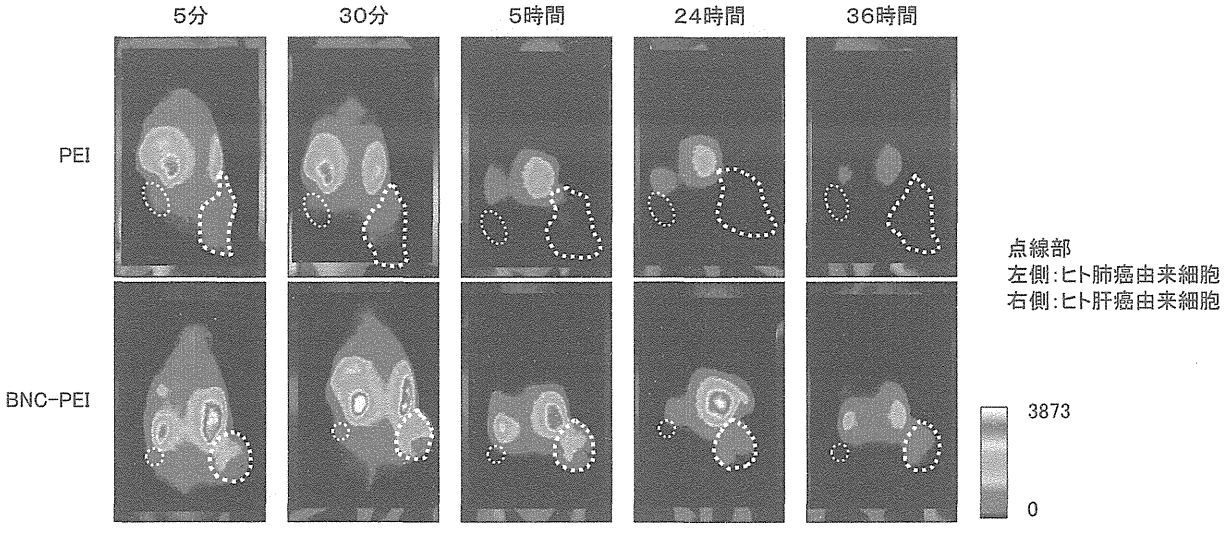

Fig. 4 BNC-PEI 複合体を用いた *in vivo* DDS 実験

ではこの肝臓特異性を改変した再標的化 BNC の創出も重要と考えており，BNC の pre-S1 領域に含まれるヒト肝臓特異的結合領域を他の組織や細胞に特異性を示す生体認識分子（抗体，タンパク質，ペプチド等）に置換することで（Fig.5A），組織特異性を改変する研究にも注力している。当初は，遺伝子工学的に pre-S から pre-S2 領域を EGF（epidermal growth factor）に置換して EGF 提示型 BNC を発現・精製し，*in vitro* においてヒト肝癌由来細胞への特異性を消失させ，EGF 受容体を著量発現する扁平上皮癌由来 A431 細胞への特異性を付与することに成功した〔21〕。しかし本法では，再標的化の度に改変型 BNC 発現プラスミドを作製せねばならず，また，置換した生体認識分子の一部が出芽酵母内での BNC の粒子形成過程を著しく阻害したことから，BNC の特徴である過剰発現が困難となった。そこで，再標的化の分子として汎用性の高い抗体に特定し，BNC の pre-S1 から pre-S2 領域を黄色ブドウ球菌由来 Protein A の IgG Fc 結合部位（Z 領域）の 2 個（ZZ）に置換した ZZ-L タンパク質提示型 BNC（zzBNC）を作製した（Fig.5B）〔21〕。zzBNC は，BNC より小さい直径約 40 nm 程のナノ粒子として出芽酵母内で過剰発現でき，BNC とほぼ同様の精製法で大量生産が可能であった。また，zzBNC は HBV のヒト肝臓細胞内部への侵入過程過で最も重要な役割を担う pre-S1 領域の膜融合活性領域（N 末端側 20 aa）を保持しているので，BNC 同様にリポソームとの融合も可能であった。抗 EGFR 抗体を提示した zzBNC を，グリオーマを同所移植したマウスに脳内投与すると，グリオーマ特異的に著量の zzBNC が蓄積した〔23〕。また，炎症部位の血管内皮細胞に誘導される E セレクチンに注目し，網膜にブドウ膜炎，足関節にリューマチを発症させたモデルマウス，また心筋梗塞モデルラットにおいて，抗セレクチン E 抗体を提示した zzBNC を静脈投与すると，速やかに各炎症部位に集積した（鄭ら，投稿中）。特にぶどう膜炎部位には蛍光ビーズの導入および GFP 遺伝子の発現に成功した。一方，zzBNC の ZZ 領域には 13 個のリジン残基が存在し，1 級アミン反応性の NHS（N-hydroxysuccinimidyl ester）化合物を用いる化学結合により，抗体以外の生体認識分子（化合物，タンパク質，ホーミングペプチドなど）の提示も容易に行えた。例えば，悪性腫瘍に高頻度で見られる糖鎖構造の β1-6GlcNac を認識する PHA-L4 レクチンを表層提示した zzBNC は，*in vitro* と *in vivo* においても β1-6GlcNac を有する悪性腫瘍へと効率よく集積し〔24〕，腫瘍内部のリンパ管壁を認識する 9 アミノ酸のホーミングペプチド（Lyp-1 ペプチド）〔25, 26〕を表層提示した zzBNC は，ヒト乳癌細胞由来組織の担癌マウスにおいて，蛍光物質や GFP 発現遺伝子の送達を可能にした。しかし，ホーミングペプチド提示型 zzBNC は，他の再標的化 BNC よりオフターゲット率も高く，再標的化分子としては不完全であり，タンパク質のような高次構造で認識する再標的化分子が望ましい。以上のように，BNC は *in vivo* において癌組織を能動的標的化する DDS キャリアとして有望と思われた。

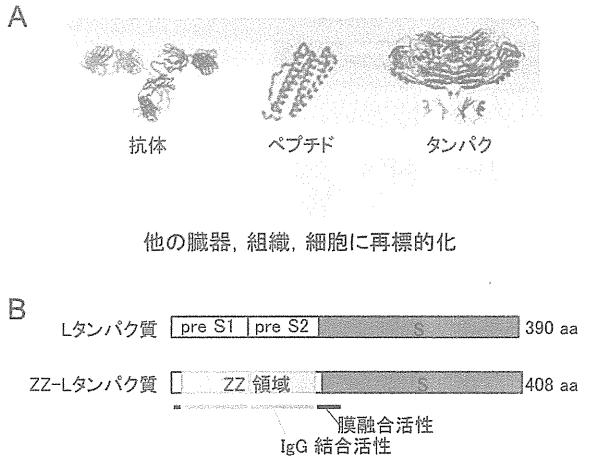

Fig. 5 BNC の再標的化

6. 新たなワクチンプラットフォームへの挑戦

以上のように，BNC は癌組織や炎症部位へ効率よく薬剤を送達できると期待できたが，固形癌への薬剤集積は EPR 効果により受動的標的化する DDS キャリアが最近の主流であり，実際に能動的標的型 DDS キャリアと受動的標的型 DDS キャリアの腫瘍組織への集積度合に大差がないことも報告されている〔27〕。そこで我々は，能動的標的型 DDS キャリアの機能を十分に活かすべく，zzBNC を用いる新たなワクチンプラットフォームの開発に着手している。従来のワクチンは，アジュバントとともに接種された抗原が近傍のリンパ節内部に受動的に移行し，免疫の中心的役割を担う抗原提示樹状細胞（DCs：dendritic cells）にバイチャンスで取り込ませて免疫系を作動させた。一方，最近のナノ粒子を活用したワクチンでは，抗原を DCs が好む直径数百 nm のナノ粒子化にして，DCs に受動的に取り込まれ易くする工夫が成されている。しかし，能動的標的型 DDS キャリアを用いて DCs に積極的に抗原を積極的に送達させ，高い免疫賦活化能を有する「攻めのワクチン」になると考えた。具体的には，DCs の細胞表面に存在する特異的抗原（CD11c, MHC class II, ガングリオシド GM1）を認識する抗体を zzBNC に提示させてマウスに尾静脈から投与し，脾臓内 DC 細胞への集積性を評価したところ，抗 CD11c 抗体を提示する zzBNC が脾内 DC 細胞の 8 割以上に zzBNC が集積していた〔28〕。次に，日本脳炎ウイルスに対するサブユニットワクチン（D3 抗原）と複合体を形成したカチオニックリポソームと抗 CD11c 抗体提示型 zzBNC を融合し，マウスに尾静脈から投与したところ，抗原単独投与より，血清中の抗 D3 抗体が飛躍的に産生されていた（Fig.6）。このような増強効果は，単純に DCs への積極的な抗原送達だけではなく，zzBNC による抗体分子の BNC 表面への整列化による avidity の増強による相乗効果と考えられる〔29〕。

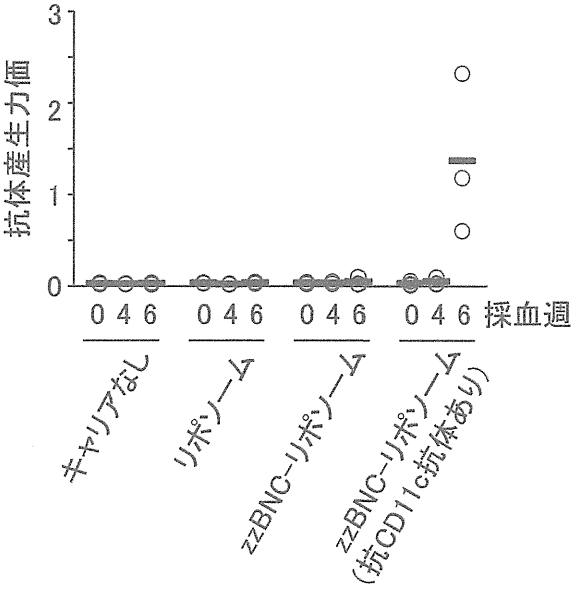

Fig. 6 BNC による効果的な抗体産生誘導

7. まとめ

HBV を模倣した BNC（ステルス型 BNC）は，血液中を RES に捕捉されることなく滞留し，肝実質細胞に特異的に取り込まれ，理想的な DDS キャリアとして機能した。これまでに我々は，BNC の細胞内侵入能は HBV に匹敵することを明らかにしており〔30〕，さらに細胞内へと侵入した後では，酸性条件下で発揮されるエンドソーム脱出能も併せもつことも報告している。これまでの DDS キャリアは，生体レベルの動態解析に基づきマクロレベルでの最適化が進められてきた一方で，細胞内および生体内解析技術の進展により，送達する薬剤の種類に従って，細胞外，細胞膜，細胞質，エンドソーム，核へとミクロレベルで細胞内動態を最適化する必要性が高まってきている。BNC についても，今後より詳細に細胞内動態を解析する必要があり，HBV が肝細胞表面のヘパラン硫酸プロテオグリカンなどの HBV 低親和性受容体と結合し，次に NTCP (Na-taurocholate cotransporting polypeptide) などの HBV 高親和性受容体〔31〕に引き渡されて，その後細胞内に侵入すると考えているが〔30〕，BNC（あるいは HBV）の細胞への侵入のメカニズムは

詳細には分かっていない。我々は，pre-S1 領域 N 末端 20 アミノ酸残基に存在する膜融合活性領域が低 pH 依存的に活性化すると考えられているが，その詳細な作用機構については明らかになっていない。さらに，遺伝子送達においては，核内へと治療遺伝子を送達させる必要があるが，核膜のポアを通過するには ATP 依存的な能動輸送タンパク質が関与しており，核酸のような巨大分子の通過は困難である。従って，BNC を用いる核酸送達においては，HBV におけるコアタンパク質のような能動的核内移行メカニズムを付与する必要があると考えており，HBV の機能を完全に引き継ぐ次世代の DDS キャリア，次世代の BNC 開発を進めている。

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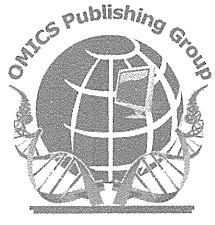

ISSN:

Journal of Infectious Diseases & Therapy

The International Open Access
Journal of Infectious Diseases & Therapy

Available online at: OMICS Publishing Group (www.omicsonline.org)

Digital Object Identifier: http://dx.doi.org/10.4172/jidt.1000e101

Infectious Diseases & Therapy

Malick et al., J Infect Dis Ther 2013, 1:1
http://dx.doi.org/10.4172/2165-7866.1000e101

Editorial Open Access

Start or End? One of the Biggest Mysteries is Finally Solved?

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Hepatitis B virus (HBV) is a small DNA virus, which was found a half century ago. No useful and convenient *in vitro* infection system for HBV has hampered detailed analysis of HBV life cycle, which leads to development of HBV related diseases. Here, Yan et al. [1] reported this November, 2012 that a sodium-taurocholate cotransporting polypeptide also called NTCP was a receptor for HBV and established an HBV *in vitro* infection system using ordinary hepatocellular carcinoma derived cell lines. This finding will shed light on total elucidation of the HBV life cycle and lead to development of new anti-HBV drugs.

It is now a half century since hepatitis B virus (HBV) was identified by Blumberg et al. [2]. This virus is a small DNA virus whose genome is partially double stranded circular DNA and is only 3.2 kb in size. The viral genes are compactly organized and four genes; core, S, polymerase and X are identified [3].

There are about 360 million people infected with the virus worldwide, forming one of the biggest infectious diseases and one of the most serious health programs of human being [4]. HBV infection causes not only severe acute hepatitis including fulminant hepatitis that is highly lethal, but also chronic hepatitis leading liver cirrhosis and hepatocellular carcinoma especially through vertical infection. Treatment against HBV infection consists of interferon therapy and anti-viral drugs. The interferon therapy is, however, very limited and not as effective as that against hepatitis C virus (HCV) infection [5]. And furthermore, anti-HBV drugs, all of which are nucleoside analogues, were developed as anti-human immunodeficiency virus (HIV) drugs not as anti-HBV ones and the HBV polymerase specificity and peculiarity were not considered. Needless to say, both viruses have a reverse transcription process of their replication cycles and thus reverse transcriptase activity work in a similar way, i.e., their activity center is composed of an MYDD motif [3]. These kinds of anti-viral drugs always lead to generation and expansion of mutants that escape their effectiveness.

As mentioned, exploitation and development of new really effective drugs against HBV considering its viral life cycle and gene functions is one of the most important issues for human health. But we have not had very useful HBV infection system *in vitro* and *in vivo* to study detail viral life cycle and its pathogenesis. Hepadnaviruses are highly species specific and tissue specific and HBV never infects present hepatocyte-originated human hepatocellular carcinoma cell lines such as HepG2. Although there are several animal hepadnaviruses; avians (duck hepatitis B virus (DHBV) and heron hepatitis B virus (HHV)), rodents (woodchuck hepatitis virus (WHV) and ground squirrel hepatitis virus (GSHV)) and primates for HBV [6,7], it is not easy to hatch and raise these kinds of animal and to prepare primary hepatocytes from these animals.

As for *in vitro* infection systems, two systems are available at present; one is primary human hepatocytes (PHH) or tree shrew (*Tupaia belangen*) [8] which is also susceptible to HBV as human and chimpanzee and another is HepaRG [9], a cultured human hepatocellular carcinoma (HCC) cell line established from HCV caused HCC. These *in vitro* systems facilitate our knowledge about how HBV infects hepatocytes, but still not good enough because of their commercialism, invariability is dependent on the lot and takes cost for preparation and time to induce infectivity.

A recent splendid report by Yan et al. may improve such inconvenience to study HBV [1]. For long time, we have been searching for HBV receptors and several molecules including those from the DHBV system were nominated as HBV receptors but they had never contributed establishment for HBV infection systems *in vitro*, though many data have suggested that the ligand on the HBV side for the receptor must be in the preS1 region [10-12]. This time, Yan et al. [1] successfully pulled down an HBV receptor, a sodium taurocholate cotransporter polypeptide called also NTCP or NTCP-1, with a photo-activating preS1 peptide using Primary Tupaia Hepatocytes (PTH). Many researchers had been trying to elucidate HBV receptors with the same kind methods including phage libraries screening with the preS1 region; synthetic peptides or the protein purified from *E. coli*, insect cells and yeast expression/purification system etc., and wonder why such a molecule has not been picked up until now.

Yan et al. [1] showed that NTCP expression was low in common HCC cell lines but high in PHH and PTH, and that introduction of NTCP expression raised the competency of HBV in these unsusceptible cell lines and knockdown of NTCP in PTH lowered the susceptibility.

Thus, a tip of the mystery has been finally solved by Yan et al. [1]. Though this must be re-evaluated by the other researchers, the finding will shed light on total elucidation of the HBV life cycle and lead to development of new anti-HBV drugs.

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Received December 20, 2012; **Accepted** December 22, 2012; **Published** December 29, 2012

Citation: Ueda K (2013) Start or End? One of the Biggest Mysteries is Finally Solved? J Infect Dis Ther 1: e101. doi:10.4172/jidt.1000e101

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Citation: Ueda K (2013) Start or End? One of the Biggest Mysteries is Finally Solved? J Infect Dis Ther 1: e101. doi:10.4172/jidt.1000e101

PLOS ONE

Inhibitory Effects of Caffeic Acid Phenethyl Ester Derivatives on Replication of Hepatitis C Virus

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Abstract

Caffeic acid phenethyl ester (CAPE) has been reported as a multifunctional compound. In this report, we tested the effect of CAPE and its derivatives on hepatitis C virus (HCV) replication in order to develop an effective anti-HCV compound. CAPE and CAPE derivatives exhibited anti-HCV activity against an HCV replicon cell line of genotype 1b with EC_{50} values in a range from 1.0 to 109.6 μM. Analyses of chemical structure and antiviral activity suggested that the length of the n-alkyl side chain and catechol moiety are responsible for the anti-HCV activity of these compounds. Caffeic acid n-octyl ester exhibited the highest anti-HCV activity among the tested derivatives with an EC_{50} value of 1.0 μM and an SI value of 63.1 by using the replicon cell line derived from genotype 1b strain Con1. Treatment with caffeic acid n-octyl ester inhibited HCV replication of genotype 2a at a similar level to that of genotype 1b irrespectively of interferon signaling. Caffeic acid n-octyl ester could synergistically enhance the anti-HCV activities of interferon-alpha 2b, daclatasvir, and VX-222, but neither telaprevir nor danoprevir. These results suggest that caffeic acid n-octyl ester is a potential candidate for novel anti-HCV chemotherapy drugs.

Citation: Shen H, Yamashita A, Nakakoshi M, Yokoe H, Sudo M, et al. (2013) Inhibitory Effects of Caffeic Acid Phenethyl Ester Derivatives on Replication of Hepatitis C Virus. PLoS ONE 8(12): e82299. doi:10.1371/journal.pone.0082299

Editor: Hak Hotta, Kobe University, Japan

Received September 15, 2013; **Accepted** October 31, 2013; **Published** December 17, 2013

Funding: This work was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare and from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Hepatitis C virus (HCV) is well known as a major causative agent of chronic liver disease including cirrhosis and hepatocellular carcinoma and is thought to persistently infect 170 million patients worldwide [1]. HCV belongs to the genus *Hepacivirus* of the family *Flaviviridae* and possesses a viral genome that is characterized by a single positive strand RNA with a nucleotide length of 9.6 kb [2]. The single polypeptide coded by the genome is composed of 3,000 amino acids and is cleaved by host and viral proteases, resulting in 10 proteins, which are classified into structural and nonstructural proteins [3]. The viral genome is transcribed by a replication complex consisting of NS3 to NS5B and host factors [4]. NS3 forms a complex with NS4A and becomes a fully active form to cleave the C-terminal parts of the nonstructural proteins. The advanced NS3/4A protease inhibitors, telaprevir and boceprevir, have been employed in the treatment of chronic hepatitis C patients infected with genotype 1 [5]. Sustained virologic response (SVR) was reportedly 80% in patients infected with genotype 1 following triple combination therapy with pegylated interferon, ribavirin, and telaprevir [6], although the therapy exhibits side effects including rash, severe cutaneous eruption, influenza-like symptoms, cytopenias, depression, and anemia [7]. In addition, there is the possibility of the emergence of drug-resistant viruses following treatment with those anti-HCV drugs [8] Thus, further study is required for development of safer and more effective anti-HCV compounds.

Several recent reports indicate that silbinin [9], epigallocatechin-3-gallate [10], curcumin [11], quercetin [12] and proanthocyanidins [13], which all originate from natural sources, have exhibited inhibitory activity against HCV replication in cultured cells. Caffeic acid phenethyl ester (CAPE) is an active component included in propolis prepared from honeybee hives, and has a similar structure to flavonoids (Fig. 1A). CAPE has multifunctional properties containing anti-inflammatory [14], antiviral [15], anticarcinogenic [16], and immunomodulatory activities [15]. CAPE also inhibits enzymatic activities of endogenous and viral proteins [17–19] and transcriptional activity of NF-kappaB [14,20]. In addition, CAPE could suppress HCV replication enhanced by using the NF-kappaB activation activity of morphine [21], although it has been unknown which of moieties including CAPE is responsible for anti-HCV activity. Furthermore, it is not clear whether chemical modification of CAPE could enhance anti-HCV activity or not. In this report, we examined the effect of

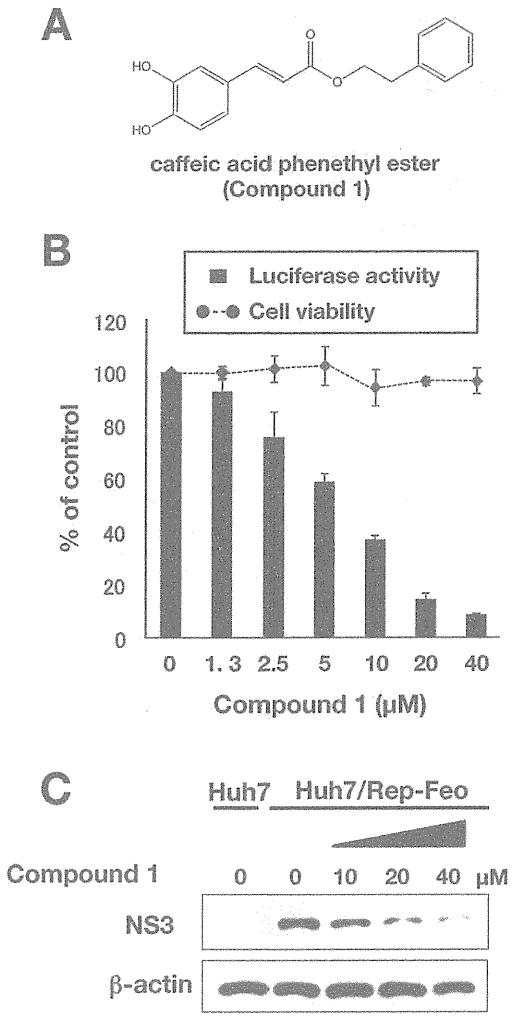

Figure 1. Effect of CAPE on viral replication in the replicon cell line of genotype 1b. (A) Molecular structure of CAPE. (B) Huh7/Rep-Feo cells were incubated for 72 h in a medium containing various concentrations of CAPE. Luciferase and cytotoxicity assays were carried out by the method described in Materials and Methods. Error bars indicate standard deviation. The data represent results from three independent experiments. (C) Protein extract was prepared from Huh7/Rep-Feo cells treated for 72 h with the indicated concentration of CAPE and it was then was subjected to Western blotting using antibodies to NS3 and beta-actin.
doi:10.1371/journal.pone.0082299.g001

CAPE derivatives on HCV proliferation to develop more effective and safer anti-HCV compounds.

Results

Effect of CAPE on HCV RNA replication in HCV subgenomic replicon cells

CAPE is composed of ester of caffeic acid and phenethyl alcohol (Fig. 1A). We examined the effect of CAPE (compound **1**) on both viral replication and cell growth in the HCV subgenomic replicon cell line Huh7/Rep-Feo. The replicon cell line was treated with various concentrations of compound **1**. The replication level of the HCV RNA was measured as an enzymatic activity of luciferase, which is bicistronically, encoded on the replicon RNA. Compound **1** suppressed HCV RNA replication at concentrations from 1.3 to 40 μM in a dose-dependent manner, but did not affect cell viability (Fig. 1B). HCV NS3, which is a viral protease, was decreased at the protein level by treatment with CAPE in a dose-dependent manner, corresponding to the viral replication, whereas beta-actin was not changed in the replicon cell line (Fig. 1C). Based on the calculation using a dose dependency of CAPE, compound **1** exhibited an EC_{50} value of 9.0 μM and a CC_{50} value of 136.1 μM, giving a selectivity index estimate (SI) of 17.9 (Table 1). These results suggest that treatment with CAPE inhibits HCV replication in HCV subgenomic replicon cells.

Structure-activity relationship of CAPE analogues

To clarify the structure-activity relationship of CAPE analogues, we examined the effect of hydroxyl groups on the aromatic ring (catechol moiety), the alkenyl moieties on alpha, beta-unsaturated esters, and the ester parts as follows (Figure S1).

We tested whether commercially available CAPE-related compounds **2** to **6** (Fig. S1) affected HCV replication (Table 1). All these compounds showed weaker inhibitory activity than CAPE (**1**), but are not toxic. Compound **2**, which is the acid component of CAPE, showed a slightly lower value of EC_{50} than compound **3**, which is the compound **2** derivative replaced a hydroxyl group with a methoxyl group of catechol moiety, while compound **4**, which is the derivative lacking two hydroxyl groups within catechol moiety, exhibits a higher value of EC_{50} than compounds **1** and **2**. These data suggest that the catechol moiety of CAPE is required for anti-HCV activity. Interestingly, compounds **5** and **6**, which are natural products including polyhydroxylated acid moieties in the ester parts, showed much weaker inhibitions than compound **1** and exhibits low *Clog P* values. The position of hydroxyl group or/and the structure of the ester part may affect the inhibitory activity and/or hydrophobicity.

We next examined the effects of caffeic acid ester compounds **7** to **11**, which include various lengths of alkyl side chains, on HCV replication (Table 2 and Figure S2). The EC_{50} values decreased in the order methyl ester (compound **7**), n-butyl ester (compound **8**), n-hexyl ester (compound **9**), and n-octyl ester (compound **10**), suggesting that elongation of the n-alkyl side chain increased the inhibitory activity. However, the EC_{50} value of n-dodecyl ester (compound **11**) was higher than that of compound **10**. Thus, n-octyl ester (compound **10**) showed the lowest EC_{50} value and the highest SI among the tested compounds shown in Tables 1 and 2. Compounds **7** to **11** gradually increased own *Clog P* values,

Table 1. Effect of CAPE (**1**) and related compounds **2–6** on HCV replication.

Compound (Number)	EC_{50} [a] (μM)	CC_{50} [b] (μM)	SI [c]	*Clog P* [d]
CAPE (**1**)	9.0±0.7	136.1±1.9	17.9	3.30
caffeic acid (**2**)	36.6±6.7	>320	>8.7	0.98
ferulic acid (**3**)	71.9±5.8	>320	>4.5	1.42
cinnamic acid henethyl ester (**4**)	86.1±6.3	>320	>3.7	4.56
chlorogenic acid (**5**)	103.0±3.4	>320	>3.1	−0.96
rosmarinic acid (**6**)	109.6±1.1	>320	>2.9	1.10

a: Fifty percent effective concentration based on the inhibition of HCV replication.
b: Fifty percent cytotoxicity concentration based on the reduction in cell viability.
c: Selectivity index (CC_{50}/EC_{50}).
d: Determined with ChemDraw software (Chem Bio Office Ultra, 2008).
doi:10.1371/journal.pone.0082299.t001

corresponding to length of n-alkyl side chain (Fig. 2A). Compounds **10** and **11** exhibit EC_{50} values of 2.7 and 5.9 μM, respectively, SI values of 29.6 and 9.80, respectively, and *Clog P* values of 4.90 and 5.96, respectively, suggesting that high hydrophobic property of n-alkyl side chain decreases anti-HCV activity. The appropriate *Clog P* value of caffeic acid ester containing unsaturated side chain may be around 5.

Dihydrocaffeic acid methyl ester (compound **12**) showed less activity than caffeic acid methyl ester (compound **7**) regardless of values of *Clog P* value and CC_{50}, suggesting that the alpha, beta-unsaturated part attached to ester affects the anti-HCV activity level (Table 3 and Figure S3).

We further examined the effect of the hydroxyl groups on the aromatic ring on HCV replication (Table 4 and Figure S4). The EC_{50} values of *O*-methylated caffeic acid n-octyl esters (compounds **13** and **14**) were higher than that of compound **10**. Compounds **15** including 3, 4-di-*O*-methylated caffeic acid n-octyl ester exhibited higher EC_{50} than values of compounds **10**, **13** and **14**. However, addition of a third hydroxyl group to 3, 4, 5-trihydroxy derivative (compound **16**) of compound **10** resulted in a reduction of anti-HCV activity. Furthermore, *Clog P* values of compound **10**, **13**, **14**, **15** and **16** were not correlated with anti-HCV activity (EC_{50} value) (Fig. 2B). These results suggest that the catechol moiety plays an important role in anti-HCV activity, and that the 4-hydroxy moiety is more important for the activity than the 3-hydroxy moiety.

Thus, compound **10**, which exhibits the lowest EC_{50} value and the highest SI value, is the most effective compound among CAPE analogues used in this study.

Effect of CAPE derivatives on virus production

The structure of compound **10** is shown in Fig. 3A. Treatment with compound **10** reduced HCV replication and NS3 protein in a dose-dependent manner at a higher anti-HCV level than

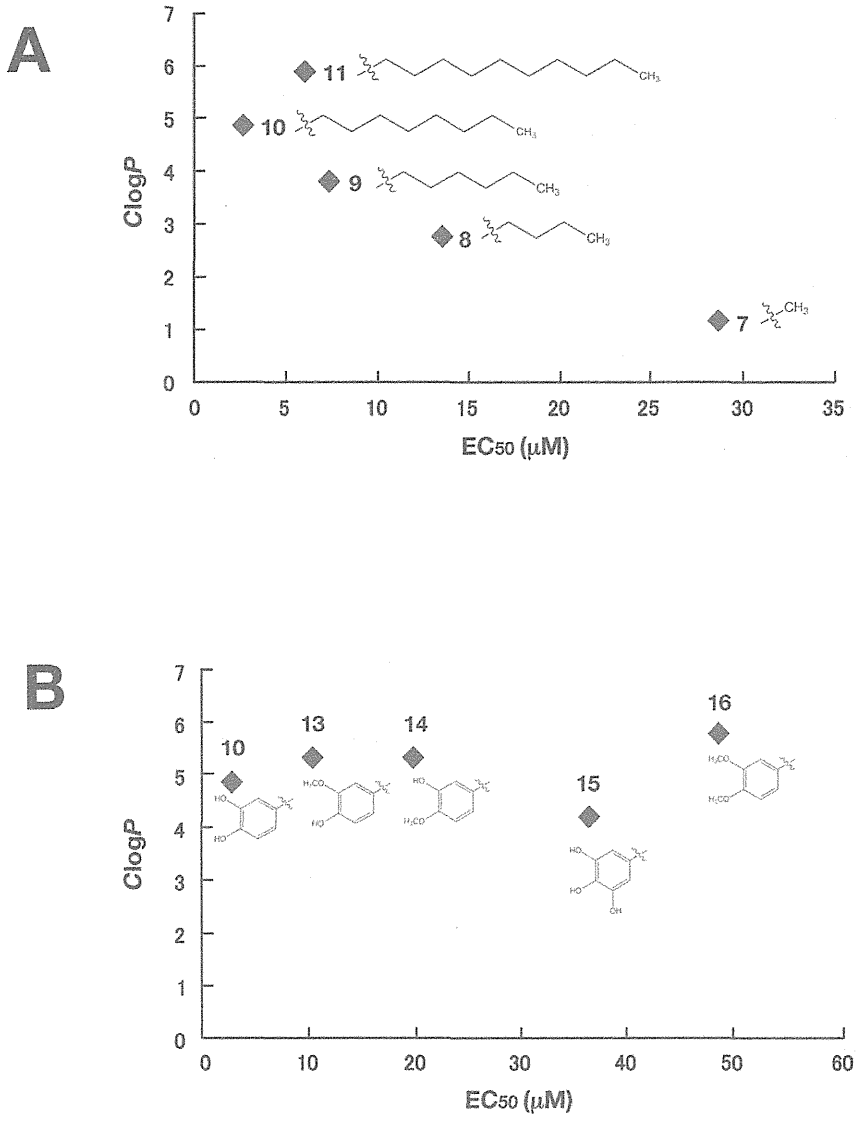

Figure 2. Correlation between the inhibitory effect on HCV replication *and* Clog *P* of CAPE analogues. Values of *x*-axis indicate EC_{50} values of CAPE analogues, while values of *y*-axis show *Clog P* values. (A) Correlation between the inhibitory effect on HCV replication and *Clog P* of CAPE analogues (Compound 7–11). (B) Correlation between the inhibitory effect on HCV replication and *Clog P* of CAPE analogues (Compound 10 and 13–16).
doi:10.1371/journal.pone.0082299.g002

Table 2. Effect of caffeic acid esters **7, 9–14**, including **1**, on HCV replication.

Compound No.	R	EC_{50} [a] (μM)	CC_{50} [b] (μM)	SI [c]	Clog *P* [d]
7	CH_3	28.6±1.2	122.1±5.0	4.2	1.20
8	C_4H_9	13.5±2.1	39.0±1.1	2.9	2.79
9	C_6H_{13}	7.3±0.2	37.6±1.2	5.1	3.85
10	C_8H_{17}	2.7±0.1	71.7±8.5	26.6	4.90
11	$C_{10}H_{21}$	5.9±0.9	57.9±2.9	9.8	5.96
1	$(CH_2)_2Ph$	9.0±0.7	136.1±1.9	17.9	3.30

The basic structure and side moieties are shown in Figure S2.
a: Fifty percent effective concentration based on the inhibition of HCV replication.
b: Fifty percent cytotoxicity concentration based on the reduction in cell viability.
c: Selectivity index (CC_{50}/EC_{50}).
d: Determined with ChemDraw software (Chem Bio Office Ultra, 2008).
doi:10.1371/journal.pone.0082299.t002

compound **1** (Figs. 3B and C), but not effect enzymatic activities of firefly and *Renilla* luciferases (Fig. 3D) and IRES-dependent translation (Fig. 3E), suggesting that inhibition of HCV replication by compound **10** is not due to offtarget effect. We evaluated the inhibitory effect of compound **10** on three different subgenomic replicon cell lines (1b: N strain, Con1 strain, 2a: JFH-1 strain) and one full genome replicon cell line (1b: O strain). Compound **10** inhibited the viral replication of all replicon cell lines at similar level, and exhibited the lowest EC_{50} value of 1.0 μM and an SI value of 63.1 by using Con1 replicon cells (Table 5). We next examined the effect of compound **10** on virus production by using HCVcc, since subgenomic replicon mimics HCV replication, but not the whole viral cycle. The Huh7 OK1 cell line, which is highly permissive to the HCV JFH1 strain [22], was infected with HCVcc and then treated with compound **10** at 24 h post-infection. The supernatant was harvested 72 h post-infection from the culture supernatant and then the RNA that prepared from the supernatant was estimated by real time qRT-PCR. Figure 3F shows that treatment with compound **10** reduced HCV viral production ($EC_{50} = 1.8 \pm 0.4$ μM) in a similar way to the data obtained by using a replicon cell line. To clarify whether or not compound **10** inhibited HCV replication via interferon-signaling pathway, we analyzed ISRE activity and the expression of interferon stimulated gene (ISG) by using reporter assay and RT-PCR, respectively. The replicon cells were harvested at 48 h post-treatment. There were no significant effects of compound **1**, **6** and **10** on ISRE-promoter activities, while interferon alpha 2b significantly enhanced it as a positive control (Fig.4 A). The data of the RT-PCR analysis showed that the transcriptional expressions of ISGs including Mx1, MxA, IFIT4, ISG15, OAS1, OAS2, and OAS3 were induced with interferon alpha 2b, but not with compound **1**, **6** and **10** (Fig. 4B). These data suggest that the CAPE derivatives have an inhibitory effect on virus production and replication, irrespective of interferon signaling induction.

Synergistic effect of caffeic acid n-octyl ester on interferon and direct-acting antiviral agents

To estimate the effects of drug combinations on anti-HCV activity, we examined the antiviral activity of compound **10** in combination with IFN-α 2b, telaprevir (NS3 protease inhibitor), danoprevir (NS3 protease inhibitor), daclatasvir (NS5A inhibitor) or VX-222 (NS5B polymerase inhibitor). Con1 LUN Sb #26 replicon cells were treated with compound **10** in combination with

Table 3. Effect of caffeic acid esters **7**and **8** on HCV replication.

Compound No.	EC_{50} [a] (μM)	CC_{50} [b] (μM)	SI [c]	Clog *P* [d]
7	28.6±1.2	122.1±5.0	4.2	1.20
12	77.0±1.6	140.7±3.4	1.8	1.02

Chemical structures of both compounds are shown in Figure S3
a: Fifty percent effective concentration based on the inhibition of HCV replication.
b: Fifty percent cytotoxicity concentration based on the reduction in cell viability.
c: Selectivity index (CC_{50}/EC_{50}).
d: Determined with ChemDraw software (Chem Bio Office Ultra, 2008).
doi:10.1371/journal.pone.0082299.t003

each anti-HCV agent at various concentration rations for 72 h. The effect of each drug combination on HCV replication was analyzed by using CalcuSyn. An explanatory diagram of isobologram was shown at a right end of lower panels of Fig. 5A as described in Materials and Methods. As shown in the resulting isobologram, all plots of the calculated EC_{90} values of compound **10** with IFN-alpha 2b, daclatasvir, or VX-222 are located under the additive line, while the plots of compound **10** with telaprevir, or danoprevir are located above the additive line and closed to the additive line (Fig. 5A). Additionally, we determined the degree of inhibition for each drug combination was analyzed as the combination index (CI) calculation at 50, 75 and 90% of effective concentrations by using CalcuSyn. An explanatory diagram was shown at a right end of lower panels of Fig. 5B as described in Materials and Methods. On the basis of the CalcuSyn analysis, the combination of compound **10** with daclatasvir exhibited strong synergistic effect on inhibition of HCV replication in the replicon cells (Fig. 5B, upper middle). The combination of compound **10** with VX-222 exhibited an additive to synergistic effect, suggesting that it trends toward synergistic (Fig. 5B, upper right), and with IFN-alpha 2b exhibited an antagonistic to synergistic effect, suggesting that it trends toward synergistic (Fig. 5B, upper left). In contrast, the combination of compound **10** with telaprevir resulted in antagonistic effect (Fig. 5B, lower left), and with danoprevir resulted in an antagonistic to additive effect, suggesting it trends toward antagonistic (Fig. 5B, lower middle). These calculated data

Table 4. Effect of octyl esters **10** and **13–16** on HCV replication.

Compound No.	R^1, R^2, R^3	EC_{50} [a] (μM)	CC_{50} [b] (μM)	SI [c]	Clog *P* [d]
10	$R^1=R^2=R^3=H$	2.7±0.1	71.7±8.5	26.6	4.90
13	$R^1=CH_3$, $R^2=R^3=H$	10.2±1.1	60.3±1.6	5.9	5.35
14	$R^1=R^3=H$, $R^2=CH_3$	19.6±0.8	59.2±1.4	3	5.35
15	$R^1=R^2=CH_3$, $R^3=H$	48.5±1.7	212.4±6.9	4.4	5.82
16	$R^1=R^2=H$, $R^3=OH$	36.3±2.9	59.8±6.9	1.6	4.24

The basic structure and side moieties are shown in Figure S4.
a: Fifty percent effective concentration based on the inhibition of HCV replication.
b: Fifty percent cytotoxicity concentration based on the reduction in cell viability.
c: Selectivity index (CC_{50}/EC_{50}).
d: Determined with ChemDraw software (Chem Bio Office Ultra, 2008).
doi:10.1371/journal.pone.0082299.t004

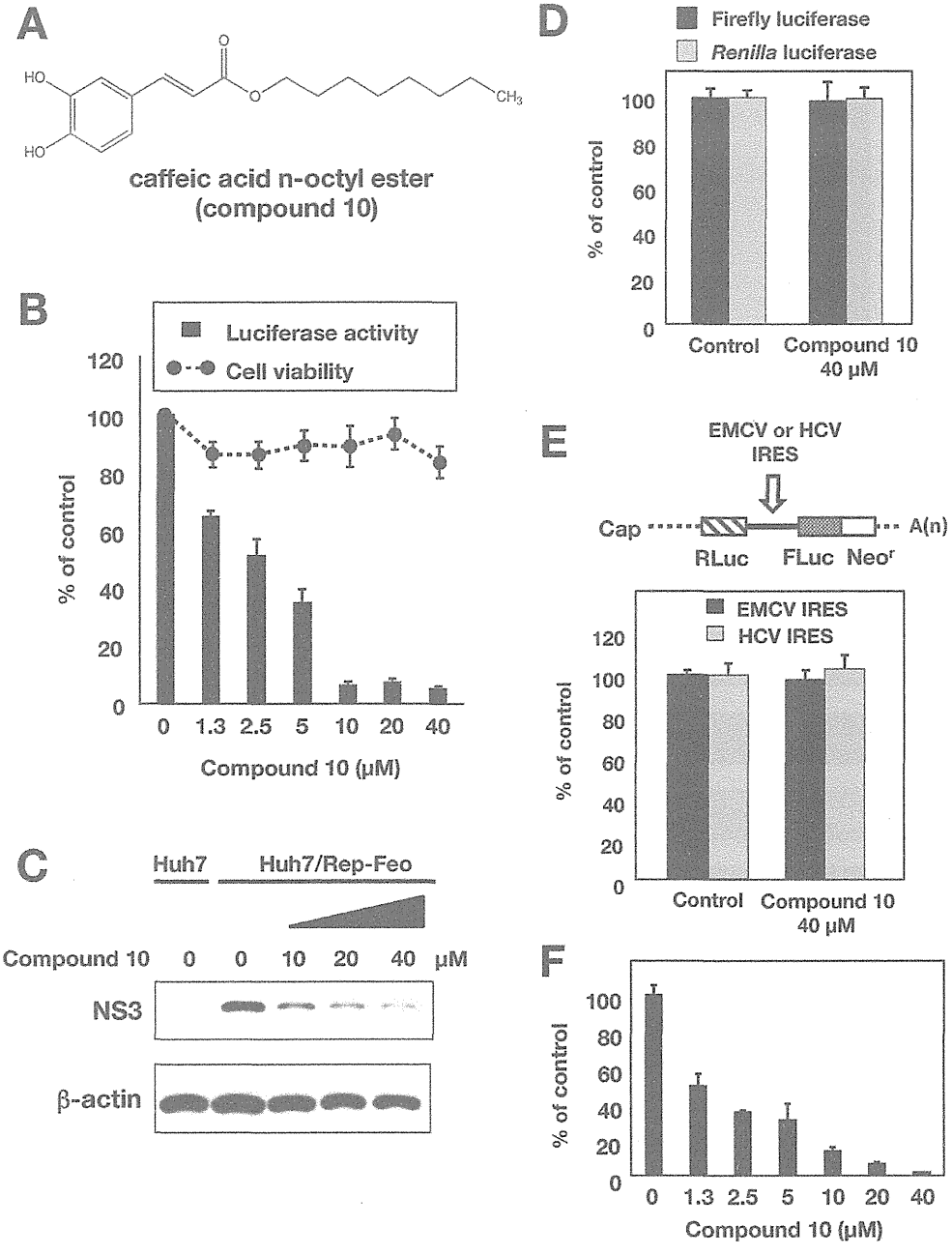

Figure 3. Effect of compound 10 on the viral replication in the replicon cell line and HCVcc. (A) Molecular structure of compound **10**. (B) Huh7/Rep-Feo cells were incubated for 72 h in a medium containing various concentrations of compound **10**. Luciferase and cytotoxicity assays were carried out by the method described in Materials and Methods. Error bars indicate standard deviation. The data represent three independent experiments. (C) Protein extract was prepared from Huh7/Rep-Feo cells treated for 72 h with the indicated concentration of compound **10** and it was then subjected to Western blotting using antibodies to NS3 and beta-actin. (D) Huh7 cell line was transfected with pEF Fluc IN encoding firefly luciferase or pEF Rluc IN encoding *Renilla* luciferase. Both transfected cell lines were incubated with DMSO (Control) or 40 μg/ml compound **10**. Firefly or *Renilla* luciferase activity was measured 72 h post-treatment. Luciferase activity was normalized with protein concentration. Error bars indicate standard deviation. The data were represented from three independent experiments. (E) Schematic structure of RNA transcribed from the plasmids was shown (Top). The bicistronic gene is transcribed under the control of elongation factor 1α (EF1α) promoter. The upstream cistron encoding *Renilla* luciferase (RLuc) is translated by a cap-dependent mechanism. The downstream cistron encodes the fusion protein (Feo), which consists of the firefly luciferase (Fluc) and neomycin phosphotransferase (Neo^r), and is translated under the control of the EMCV or HCV IRES. Huh7 cell line was transfected with each plasmid and incubated for 72 h post-treatment with DMSO (control) or 40 μg/ml of compound **10**. Firefly and *Renilla* luciferase activities were measured. Relative ratio of Firefly luciferase activity to *Renilla* luciferase activity was represented as percentage of the control condition. Error bars indicate standard deviation. The data were represented from three independent experiments. (F) Huh7 OK1 cell line was infected with HCVcc derived from JFH-1 strain and then treated with several concentrations of compound **10** at 24 h post-infection. The resulting cells were harvested 72 h post-infection. The viral RNA of supernatant was purified and estimated by the method described in Materials and Methods. Error bars indicate standard deviation. The data represent three independent experiments. Treatment with DMSO corresponds to '0'.
doi:10.1371/journal.pone.0082299.g003

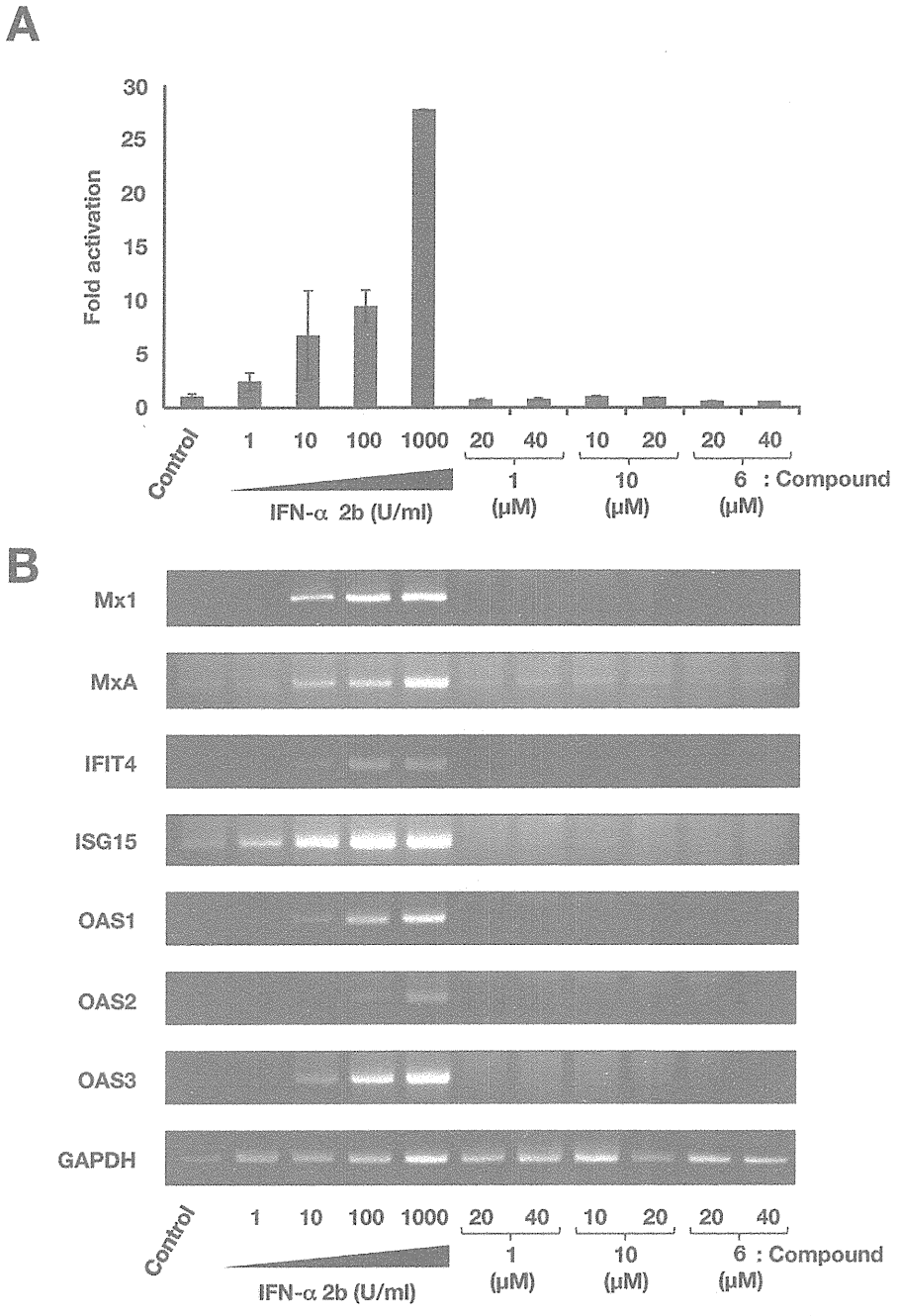

Figure 4. Effect of CAPE derivatives on the interferon-signaling pathway. (A) Plasmids pISRE-TA-Luc and phRG-TK were co-transfect into Huh7 OK1 cells. The transfected cells were cultured with 1, 10, 100, or 1000 U/mL of interferon-alpha 2b, and compounds **1**, **6** and **10**. Treatment with DMSO corresponds to '0'. After 48 h of treatment, luciferase activities were measured, and the value were normalized against *Renilla* luciferase activities. Error bars indicate standard deviation. The data represent three independent experiments. (B) Huh7 replicon cell line of genotype 1b was treated with 1, 10, 100, or 1000 U/mL of interferon-alpha 2b, and compounds **1**, **6** and **10** for 48 h. Treatment with DMSO corresponds to the control. The mRNAs of Mx1, MxA, IFIT4, ISG15, OAS1, OAS2, OAS3, and GAPDH as an internal control were detected by RT-PCR.
doi:10.1371/journal.pone.0082299.g004

of combination tests suggest that daclatasvir, IFN-alpha 2b, and VX-222 synergistically, but telaprevir and danoprevir antagonistically, inhibit HCV replication in combination with compound **10**.

Discussion

CAPE is an active component of propolis, which possesses broad-spectrum biological activities [14–19]. In this study, CAPE suppressed HCV RNA replication in a dose-dependent manner (Fig. 1A and B). Treatment with CAPE inhibited HCV replication with an EC_{50} of 9.0 μM and an SI of 17.9 in Huh7/Rep-Feo cells (Table 1). The treatment of the replicon cell line with CAPE did not induce expression of the IFN-inducible gene (Fig. 4), suggesting that the inhibition of HCV replication by CAPE is independent of the IFN signaling pathway.

Table 5. Anti-HCV activity of compound **10** in replicon cell lines of genotypes 1b and 2a.

Cell line	Replicon type	Strain (Genotype)	EC_{50} [a] (μM)	CC_{50} [b] (μM)	SI [c]
Huh7 Rep/Feo-1b	Subgenome	N (1b)	2.7±0.1	71.7±8.5	26.6
Con1 LUN Sb #26	Subgenome	Con1 (1b)	1.0±0.1	63.1±3.1	63.1
Huh7 Rep/Reo-2a	Subgenome	JFH1 (2a)	1.0±0.3	60.0±2.3	60.0
OR6	Full genome	O (1b)	1.5±0.4	61.7±0.6	41.1

a: Fifty percent effective concentration based on the inhibition of HCV replication.
b: Fifty percent cytotoxicity concentration based on the inhibition of HCV replication.
c: Selectivity Index (CC_{50}/EC_{50}).
doi:10.1371/journal.pone.0082299.t005

We also examined the effect of CAPE derivatives on HCV replication. Our data suggest that the n-alkyl side chain and catechol moiety of the CAPE derivative are critical in its anti-HCV activity (Tables 2 and 3). The EC_{50} value of the derivative decreased dependently on the length of the n-alkyl side chain until reaching octyl ester length (Table 2), while longer chains than octyl ester of a derivative led to an increase in the EC_{50} value and *Clog P* value. Compound **10**, Caffeic acid n-octyl ester, exhibited the highest anti-HCV activity among the tested compounds with an EC_{50} value of 2.7 μM and an SI value of 26.6. Cyclosporine A and its analogues could suppress the viral replication of genotype 1b at a higher level than that of genotype 2a [23]. Interestingly, compound **10** could inhibit HCV replication of genotype 1b and 2a at a similar level, irrespective of expression of the interferon-inducible gene (Fig. 4). CAPE and its derivatives may therefore possess a mechanism different from cyclosporine A and its analogues with respect to anti-HCV activity.

CAPE has been reported to be an inhibitor of NF-kappaB [14,20]. Lee et al. reported that the catechol moiety in CAPE was important for inhibition of NF-kappaB activation [24]. The data shown in Table 3 suggest that the catechol moiety in CAPE is critical to the anti-HCV activity of compound **10**. Previous studies have implicated the inhibition of NF-kappaB in anti-HCV activity. Treatment with an extract prepared from *Acacia confusa* [25] or San-Huang-Xie-Xin-Tang [26] could suppress HCV replication and inhibit NF-kappaB activation. However, Chen et al. reported that curcumin-mediated inhibition of NF-kappaB did not contribute to anti-HCV activity [11]. Furthermore, treatment with *N'*-(Morpholine-4carbonyloxy)-2(naphthalene-1-yl) acetimidamide could activate NF-kappaB and downstream gene expression in the same Huh7/Rep-Feo replicon cell line as the cell line used in this study and exhibited potent inhibition of HCV replication without interferon signaling [27]. These reports support the notion that CAPE derivatives do not mainly target NF-kappaB activity as part of their anti-HCV activity.

Several host proteins have been reported to regulate function of NS5A, leading to supporting HCV replication (review in [2,28]). Daclatasvir exhibited potent synergistic effect on anti-HCV activity in combination of compound **10** (Fig. 5). Anti-HCV activity of compound **10** might associate with intrinsic functions of host factors that interact with NS5A. NS3 protease inhibitors exhibited antagonistic effect in combination of compound **10** (Fig. 5). The inhibitory effect of compound **10** might be mediated by the activation of an unknown endogenous protease that is nonspecifically suppressed by NS3 protease inhibitors. Further study to clarify the mechanism by which compound **10** suppresses HCV replication might contribute to identification of a novel host factor as a drug target for development of the effective compound supporting an effect of other anti-HCV drugs.

In conclusion, we showed that CAPE and its analogue possess a significant inhibitory effect against HCV replication. The length of n-alkyl side chains and the catechol moiety of CAPE are critical to its inhibitory activity against HCV replication. The most effective derivative among the tested compounds was caffeic acid n-octyl ester, which exhibited an EC_{50} value of 1 μM and an SI value of 63.1 in the replicon cell line of genotype 1b strain Con1. Treatment with caffeic acid n-octyl ester reduced the viral replication of genotype 1b and 2a at a similar level and inhibited viral production of HCVcc. Treatment with caffeic acid n-octyl ester could synergistically enhance the anti-HCV activities of IFN-α 2b, daclatasvir, and VX-222, but neither telaprevir nor danoprevir. Further investigation to clarify the mechanism of anti-HCV activity and further modification of the compound to improve anti-HCV activity will lead to novel therapeutic strategies to treat chronic hepatitis C virus infection.

Materials and Methods

Compounds

Boldface numbers in this text indicate the compound numbers shown in Tables. All chemical structures of compounds used in this study are shown in figure S1. CAPE (**1**), caffeic acid (**2**), ferulic acid (**3**), and chlorogenic acid (**5**) and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cinnamic acid phenethyl ester (**4**) was from Tokyo Chemical Industry (Tokyo, Japan). Rosmarinic acid (**6**) was from Wako Pure Chemical (Tokyo, Japan). Caffeic acid n-octyl ester (n-octyl caffeate) (**10**), 3-O-methylcaffeic acid n-octyl ester (n-octyl-3-methylcaffeate) (**13**), 4-O-methylcaffeic acid n-octyl ester (n-octyl-3-methylcaffeate) (**14**), and 3, 4-O-dimethyl-caffeic acid n-octyl ester (n-octyl-3, 4-methylcaffeate) (**15**) were from LKT Laboratories (St. Paul, MN, USA).

Caffeic acid esters **7**, **8**, **9**, and **11** were synthesized by preparing caffeic acid chloride followed by treatment with corresponding alcohols [29]. Dihydrocaffeic acid ester **12** was prepared by hydrogenation of **7**. Compound **16** is a newly synthesized ester. Spectroscopic data of known esters **7–9**, and **11** prepared here were identical to those reported [30-32] Interferon alfa-2b (IFN-α 2b) was obtained from MSD (Tokyo, Japan). Telaprevir and daclatasvir were purchased from Selleckchem (Houston, TX, USA). Danoprevir and VX-222 were from AdooQ BioScience (Irvine, CA, USA).

Chemistry of 3,4,5-Trihydroxycinnamic acid n-octyl ester

3,4,5-Trihydroxycinnamic acid n-octyl ester (**16**) was prepared by condensation of corresponding benzaldehydes with malonic acid n-octyl monoester [33]. A solution of malonic acid n-octyl monoester (432 mg, 2 mmol), 3,4,5-trihydroxybenzaldehyde (462 mg, 3 mmol) and piperidine (0.2 mL) in pyridine (2 mL)

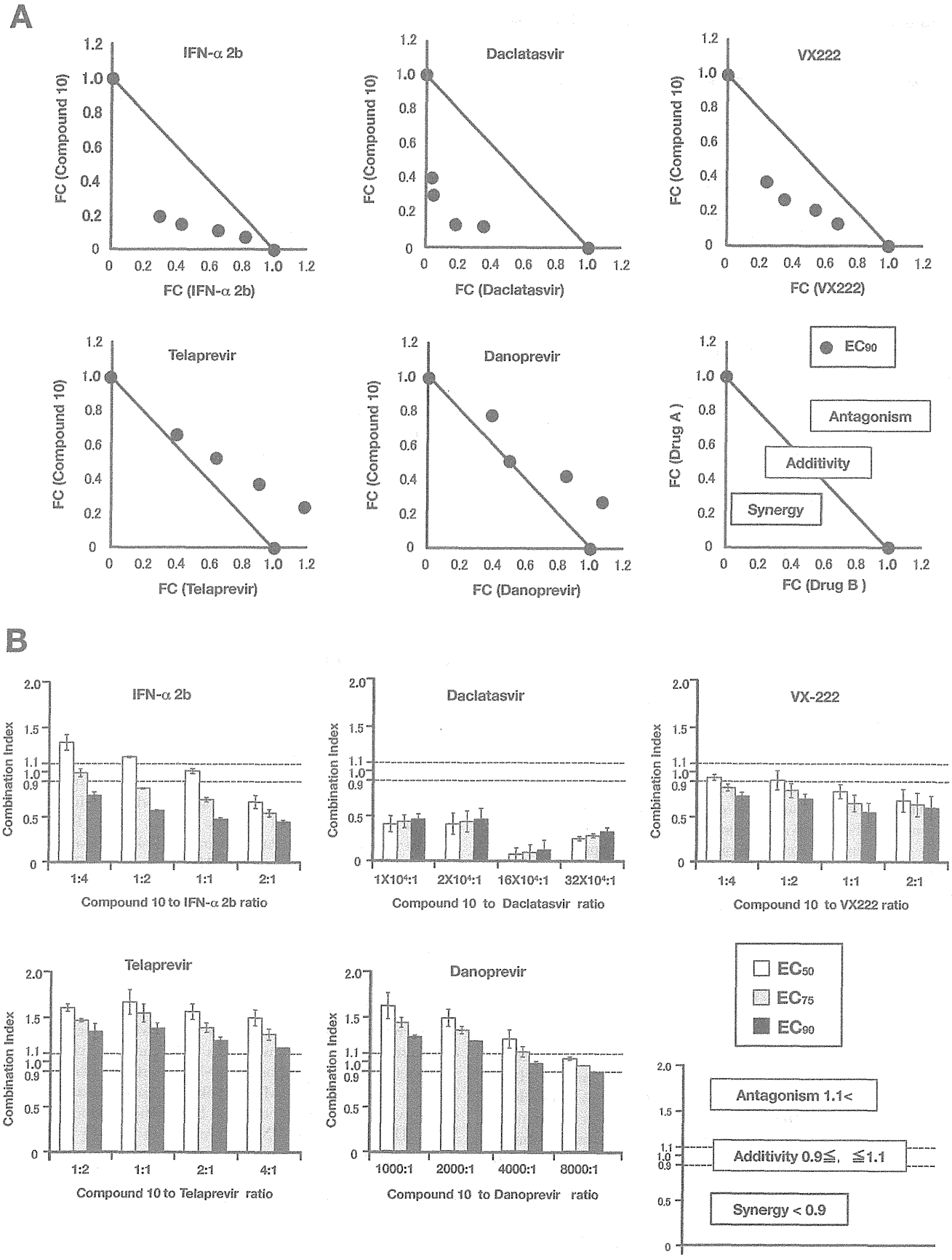
A
IFN-α 2b
FC (Compound 10)
FC (IFN-α 2b)
Daclatasvir
FC (Daclatasvir)
VX222
FC (VX222)
Telaprevir
FC (Telaprevir)
Danoprevir
FC (Danoprevir)
EC90
Antagonism
Additivity
Synergy
FC (Drug A)
FC (Drug B)
B
IFN-α 2b
Combination Index
1:4
1:2
1:1
2:1
Compound 10 to IFN-α 2b ratio
Daclatasvir
1X10⁴:1
2X10⁴:1
16X10⁴:1
32X10⁴:1
Compound 10 to Daclatasvir ratio
VX-222
Compound 10 to VX222 ratio
Telaprevir
4:1
Compound 10 to Telaprevir ratio
Danoprevir
1000:1
2000:1
4000:1
8000:1
Compound 10 to Danoprevir ratio
EC50
EC75
EC90
Antagonism 1.1<
Additivity 0.9≦, ≦1.1
Synergy < 0.9

Figure 5. Synergistic effect analyses for the combination of compound 10 with IFN-α 2b, daclatasvir, VX-222, telaprevir, or danoprevir. The Huh7 cell line, including the subgenomic replicon RNA of genotype 1b strain Con1, was treated for 72h with combinations of compound 10 and IFN-α 2b, daclatasvir, VX-222, telaprevir, or danoprevir. Luciferase assay were carried out as described in Materials and Methods. (A) The calculated EC_{90} values for combination were plotted as the fractional concentration (FC) of compound 10 and one of IFN-α 2b, daclatasvir, VX-222, telaprevir, and danoprevir on the *x* and *y* axes, respectively. Synergy, antagonism and additivity are indicated in a representative graph as a right end of lower graphs and are described in Materials and Methods. (B) Combination indexes of compound 10 with IFN-α 2b, daclatasvir, VX-222, telaprevir, or danoprevir at the EC50, EC75, and EC90 values were measured at various drug rations. Synergy, antagonism and additivity are indicated in a representative graph as a right end of lower graphs and are described in Materials and Methods.
doi:10.1371/journal.pone.0082299.g005

was heated at 70°C for 1 h. The reaction mixture was concentrated under a vacuum to give a residue, which was dissolved in $CHCl_3$-IPA (3:1, v/v) and then washed with 10% HCl and water. The organic layer was dried over Na_2SO_4 and evaporated to give a residue, which was purified by silica gel column chromatography using AcOEt-hexane (1:1, v/v) as eluent to give the corresponding n-octyl ester (85 mg, 13.8%) as a pale powder. FT-IR νmax (KBr): 3389, 3239, 2923, 1675, 1627, 1606 cm^{-1}. ^1H NMR (400MHz, CD_3OD) δ: 0.86 (3H, t, J= 7.2 Hz), 1.20–1.40 (10H, m), 1.65 (2H, quintet, J= 6.4 Hz), 4.11, (2H, t, J= 6.4 Hz), 6.16 (2H, d, J= 15.6 Hz), 6.55 (2H, s), 7.40 (2H, d, J= 15.6 Hz). 13C NMR (100 Hz, CD_3OD) δ: 14.4, 23.7, 27.1, 29.8, 30.3, 30.4, 32.9, 65.6, 108.5, 115.3, 126.6, 137.5, 147.1, 169.4. CI MS *m/z*: 309 (M^++H). High-resolution CI MS calcd. for $C_{17}H_{25}O_5$ (M^++H) for 309.1702. Found: 309.1686.

Replicon cell lines and virus infection

The Huh7/Rep-Feo cell line, which harbors the subgenomic replicon RNA composed of HCV IRES, the gene of the fusion protein consisting of neomycin phosphotransferase and firefly luciferase, EMCV IRES and a nonstructural gene of genotype 1b strain N in order in Huh7 cell line, was previously established [34]. Thus, the luciferase activity corresponds to the level of HCV RNA replication. The cell line was maintained in Dulbecco's modified Eagle medium containing 10% fetal calf serum and 0.5 mg/mL G418 and cultured in absence of G418 when they were treated with compounds. The Lunet/Con1LUN Sb #26 cell line, which harbors the subgenomic replicon RNA of the Con1 strain (genotype 1b), was described previously [35]. The OR6 cell line, which harbors the full genomic replicon RNA of the O strain (genotype 1b), was described previously [36]. The HCV replicon cell line derived from the genotype 2a strain JFH1 was described previously [37]. The viral RNA derived from the plasmid pJFH1 was transcribed and introduced into Huh7OK1 cells according to the method of Wakita et al. [38]. The virus was amplified by the several times passages. The cells were infected with the virus at a multiplicity of infection (moi) of 1 and then treated with each compound at 24 h post-infection. The culture supernatants were harvested 72 h post-treatment to estimate the viral RNA as described below.

Determination of luciferase activity in HCV replicon cells

The replicon cells were seeded at 2×10^4 cells per well in a 48-well plate 24 h before treatment. Compounds were added to the culture medium to give various concentrations. The resulting cells were harvested 72 h post-treatment and lysed with cell culture lysis reagent (Promega, Madison, WI). The luciferase activity of each cell lysate was estimated using a luciferase assay system (Promega). The resulting luminescence was detected by a Luminescencer-JNR AB-2100 (ATTO, Tokyo, Japan).

Determination of Cytotoxicity in HCV replicon cells

The replicon cells were seeded at a density of 1×10^4 cells per well in a 96-well plate and then incubated at 37°C for 24 h. Compounds were added to the culture medium to give various concentrations and were then harvested 72 h post-treatment. Cell viability was measured using a dimethylthiazol carboxymethoxyphenylsulfophenyl tetrazolium (MTS) assay with a CellTiter 96 aqueous one-solution cell proliferation assay kit (Promega).

Western Blotting

Western blotting was carried out by the method described previously [39]. The antibodies to NS3 (clone 8G-2, mousemonoclonal, Abcam, Cambridge, UK), and beta-actin were purchased from Cell Signaling Technology (rabbit polyclonal, Danvers, MA, USA) and were used as the primary antibodies in this study.

RNA analysis

Total RNAs were prepared from cells by using the RNAqueous-4PCR kit (Life Technologies, Carlsbad, CA). Viral RNA were prepared from culture supernatants by using the QIAamp Viral RNA mini kit (QIAGEN, Hilden, Germany). The viral RNA genome was estimated by the qRT-PCR method described previously [40]. RT-PCR was carried out by the method described previously [41] which was slightly modified at the PCR step. The PCR samples were incubated once for 10 min at 95°C for an initial activation step of the AmpliTaq Gold DNA Polymerase (Life Technologies), and then subjected to an amplification step of 30 repeats of the cycle consisting of three segments as follow: 0.5 min at 95°C, 1 min at 55°C and 1 min at 72°C. The primers used in this study were as follows: Mx1: 5′-AGCCACTGGACTGACGACTT-3′ and 5′-GAGGGCTGAAAATCCCTTTC-3′;

MxA: 5′-GTCAGGAGTTGCCCTTCCCA-3′ and 5′-ATTCCCATTCCTTCCCCGG-3′;

IFIT4: 5′-CCCTTCAGGCATAGGCAGTA-3′ and 5′-CTCCTACCCGTCACAACCAC -3′; ISG15: 5′-CGCAGATCACCCAGAAGAT-3′ and 5′-GCCCTTGTTATTCCTCACCA-3′;

OAS1: 5′-CAAGCTCAAGAGCCTCATCC-3′ and 5′-TGGGCTGTGTTGAAATGTGT-3′;

OAS2: 5′-ACAGCTGAAAGCCTTTTGGA-3′ and 5′-GCATTAAAGGCAGGAAGCAC-3′;

OAS3: 5′-CACTGACATCCCAGACGATG-3′ and 5′-GATCAGGCTCTTCAGCTTGGC-3′;

GAPDH: 5′-GAAGGTGAAGGTCGGAGTC and 5′- GAAGATGGTGATGGGATTTC-3′

Effects on activities of internal ribosome entry site (IRES) and luciferases

Huh7 OK1 cells were transfected with pEF.Rluc.HCV.IRES.-Feo or pEF.Rluc.EMCV.IRES.Feo [39]. These transfected cells were seeded at 2×10^4 cells per well in a 48-well plate 24 h before treatment, treated with DMSO or compound **10**, and then harvested at 72 h post-treatment. The firefly luciferase activities were measured with a luciferase assay system (Promega). The total protein concentration was measured using the BCA Protein Assay Reagent Kit (Thermo Scientific, Rockford, IL, USA) to normalize