A novel method for oral delivery of apolipoprotein mimetic peptides synthesized from all L-amino acids

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Abstract **Administered subcutaneously, D-4F or L-4F are equally efficacious, but only D-4F is orally efficacious because of digestion of L-4F by gut proteases. Orally administering niclosamide (a chlorinated salicylanilide used as a molluscicide, antihelminthic, and lampricide) in temporal proximity to oral L-4F (but not niclosamide alone) in apoE null mice resulted in significant improvement ($P < 0.001$) in the HDL-inflammatory index (HII), which measures the ability of HDL to inhibit LDL-induced monocyte chemotactic activity in endothelial cell cultures. Oral administration of L-[113-122]apoJ with niclosamide also resulted in significant improvement ($P < 0.001$) in HII. Oral administration of niclosamide and L-4F together with pravastatin to female apoE null mice at 9.5 months of age for six months significantly reduced aortic sinus lesion area ($P = 0.02$), en face lesion area ($P = 0.033$), and macrophage lesion area ($P = 0.02$) compared with pretreatment, indicating lesion regression. In contrast, lesions were significantly larger in mice receiving only niclosamide and pravastatin or L-4F and pravastatin ($P < 0.001$). In vitro niclosamide and L-4F tightly associated rendering the peptide resistant to trypsin digestion. Niclosamide itself did not inhibit trypsin activity. The combination of niclosamide with apolipoprotein mimetic peptides appears to be a promising method for oral delivery of these peptides.**—Navab, M. P., Ruchala, A. J. Waring, R. I. Lehrer, S. Hama, G. Hough, M. N. Palgunachari, G. M. Anantharamaiah, and A. M. Fogelman. **A novel method for oral delivery of apolipoprotein mimetic peptides synthesized from all L-amino acids.** ***J. Lipid Res.*** **2009.** 50: **1538–1547.**

Supplementary key words atherosclerosis • apolipoprotein A-I • apolipoprotein A-I mimetic peptides • lipoproteins • HDL

We previously reported that when the apolipoprotein A-I (apoA-I) mimetic peptide 4F was synthesized from all L-amino acids and administered orally to mice, it was ineffective in improving lipoprotein inflammatory properties (1). In contrast, when the peptide was synthesized from all D-amino acids and administered orally, lipoprotein inflammatory properties were improved and lesions were decreased in these mouse models of atherosclerosis (1). Oral administration of the 4F peptide synthesized from all L-amino acids resulted in degraded peptide, whereas the oral administration of the peptide synthesized from all D-amino acids resulted in intact peptide in the circulation (1).

When D-4F and L-4F were directly compared after subcutaneous injection in cholesterol-fed rabbits, the peptides were found to be equivalent in their ability to inhibit atherosclerotic lesions (2). Therefore, it appeared likely that D-4F and L-4F only differed in their ability to resist enzymatic degradation after oral administration. Based on the work of Garber et al. (3), we would predict that resistance to enzymatic degradation would be an advantage for oral administration of a peptide synthesized from D-amino acids, but after absorption by any route, it would likely remain undegraded in tissues for prolonged periods. As injected L-4F was equally efficacious to injected D-4F in reducing atherosclerosis and inflammation (as measured by serum amyloid A levels) (2), we set out to find a method that would allow oral delivery of L-4F. Quite by accident, we discovered that if L-4F and niclosamide were coadministered orally in a mouse model of atherosclerosis, L-4F retained its biologic activity. Niclosamide is a drug that has been in clinical use for more than three decades for the treatment of tapeworm and other parasitic infections (4). It is a salicylanilide (**Fig. 1**) with low toxicity for mammals. The studies reported here suggest that niclosamide interacts with L-4F to protect the peptide from tyrpsin digestion, thus allowing its absorption.

This work was supported in part by U.S. Public Health Service Grants HL-30568 and HL-34343, and the Laubisch, Castera, and M. K. Grey Funds at the University of California.

Manuscript received 21 October 2008 and in revised form 12 January 2009 and in re-revised form 17 February 2009.

Published, JLR Papers in Press, February 18, 2009
DOI 10.1194/jlr.M800539-JLR200

Abbreviations: apoA-I, apolipoprotein A-I; HII, HDL-inflammatory index; LPS, bacterial lipopolysaccaride; TFA, trifluroacetic acid; FTIR, Fourier transform infrared; ATR, attenuated total reflectance.

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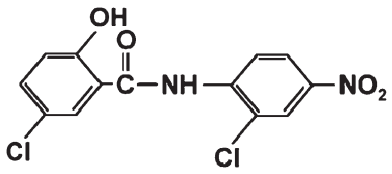

Fig. 1. Structure of niclosamide.

L-4F is a class A amphipathic helical peptide. We previously reported that a class G* amphipathic peptide taken from the sequence of apo J (residues 113-122) and synthesized from all D-amino acids (D-[113-122]apoJ) had biologic activity after oral administration similar to D-4F (i.e., a similar ability to improve lipoprotein inflammatory properties and ameliorate atherosclerosis) (5). As also reported in this manuscript, administration of niclosamide with the [113-122]apoJ peptide synthesized from all L-amino acids (L-[113-122]apoJ) resulted in biologic activity after oral administration, suggesting that coadministering niclosamide maybe a general method for oral administration of apolipoprotein mimetic peptides synthesized from L-amino acids.

METHODS

Materials

Niclosamide was purchased from Sigma-Aldrich, St. Louis, MO (Catalogue Number N3510). The structure of niclosamide is shown in Fig. 1. Trypsin was purchased from Pierce (Catalogue Number 20233). All other materials were from previously reported sources (2). L-4F and D-4F were synthesized from all L- or all D-amino acids as previously described (2). The sequence for 4F is Ac-D-W-F-K-A-F-Y-D-K-V-A-E-K-F-K-E-A-F-NH2. As in all of our previous studies, this peptide was blocked at both the C- and N- termini. For the synthesis of ^{14}C-L-4F, in the last step of the synthesis when the peptide was on the resin, ^{14}C-acetic acid was used for the acetylation. Residual free N-terminal blocking was completed with an excess of cold acetic acid. The rest of the procedures were not altered, and the radioactive peptide (like the nonradioactive peptide) was blocked at both the C- and N- termini. L-[113-122]apoJ was synthesized from all L-amino acids as previously described (5). The sequence for [113-122]apoJ is Ac-L-V-G-R-Q-L-E-E-F-L-NH2. This peptide was also blocked at both the C- and N- termini as was the case previously for D-[113-122]apoJ (5).

Mice

ApoE null mice, originally purchased from Jackson laboratories on a C57BL/6J background, were obtained from the breeding colony of the Department of Laboratory and Animal Medicine at the David Geffen School of Medicine at UCLA. The mice were maintained on a chow diet (Ralston Purina). To obtain blood for assays, the mice were subjected to a terminal bleed in which 250 μl of blood was removed from each mouse from the retro-orbital sinus under mild isoflurane anesthesia into heparinized capillary tubes and chilled plasma separator tubes (Becton-Dickson). All experiments were performed using protocols approved by the Animal Research Committee at UCLA.

Lipoprotein inflammatory indexes

Mouse plasma was obtained from heparinized blood and was sucrose-cyropreserved by adding 20% by volume of a 5× stock of filter sterilized 50% w/v sucrose, 150 mM NaCl, 0.24 mM EDTA, pH 7.4 in pyrogen-free water. The plasma was frozen and stored at –80°C until it was separated by fast protein liquid chromatography (FPLC) as previously described (6). Briefly, the column was eluted with an isocratic buffer containing 154 mmol/L NaCl, and 0.02% sodium azide, pH 8.2, at a flow rate of 0.5 ml/min, pumped by a nonmetallic Beckman high-performance liquid chromatography (HPLC) pump. Forty-eight 1-ml fractions were collected. Cholesterol concentrations were determined in fractions #13–36 to get a complete profile of the void volume through the postHDL fractions. The cholesterol profile was used to identify the apoB-containing (VLDL and LDL) and nonapoB-containing (HDL) regions. For LDL, fractions #18–23 or fractions #19–24 were pooled depending on the FPLC cholesterol profile. For HDL, fractions #25–31 or fractions #25–32 or fractions #26–32 were pooled depending on the cholesterol profile. The LDL and HDL fractions were collected and tested in cultures of human aortic endothelial cells as described previously (2). Briefly, a standard control human LDL prepared by ultracentrifugation of the plasma of a healthy volunteer was added as an internal standard to all cultures at a concentration of 100 μg/ml cholesterol. After 8 h the supernatants were collected and the monocyte chemotactic activity (which is largely due to the activity of monocyte chemoattractant protein-1) in the supernatant was determined as previously described (1, 7). The values for the control internal standard LDL were normalized to 1.0. For determination of the HDL-inflammatory index (HII), a standard control human HDL prepared by ultracentrifugation of the plasma of a healthy volunteer or mouse HDL prepared by FPLC was added at 50 μg/ml cholesterol together with the control human internal standard LDL at 100 μg/ml cholesterol. Monocyte chemotactic activity was measured as migrated monocytes per high-powered field, in triplicates in six separate fields after incubation of the endothelial cells with the lipoproteins. The value obtained by addition of the control human internal standard LDL together with the test HDL was divided by the monocyte chemotactic activity obtained after adding this LDL to the endothelial cells without HDL. In this assay anti-inflammatory HDL results in HII values less than 1.0, and pro-inflammatory HDL results in HII values greater than 1.0. For determination of the LDL-inflammatory index (LII), the test mouse LDL was added to the cells at 100 μg/ml cholesterol without added HDL, and the resulting monocyte chemotactic activity was divided by the monocyte chemotactic activity obtained after addition of the control human internal standard LDL at 100 μg/ml cholesterol without added HDL. In this assay, if the test LDL induces more monocyte chemotactic activity than the control human internal standard LDL, the LII value will be greater than 1.0. Conversely, if the test LDL produces less monocyte chemotactic activity than the control human internal standard LDL, the LII value will be less than 1.0. For both the HII and LII assay, the value for monocyte chemotactic activity of wells containing endothelial cells without addition of lipoproteins was subtracted. As shown in Results, treatment modalities in some instances reduced the monocyte chemotactic activity to below that seen with no addition of lipoproteins and resulted in negative numbers. In most instances, blood for this assay was obtained from mice 6–8 h after administration of a test agent as this time was previously found to give a near maximal response for the lipoprotein inflammatory index (1, 7).

Pre-β HDL

Pre-β HDL levels were determined using two-dimensional gel electrophoresis. Plasma was fractionated by agarose electrophoresis in the first dimension and native PAGE in the second dimension, subjected to Western analysis with anti-mouse apoA-I, and then scanned and quantified as previously described (6).

Paraoxonase activity

Paraoxonase activity was determined as described previously (6) using paraoxon as a substrate. The wells contained 1.0 mM paraoxon in 20 mM Tris/HCl, pH 8.0. The reaction was initiated by the addition of the plasma or lipoprotein sample, and the increase in the absorbance at 405 nm was recorded over a 12-min period. Blanks were included to correct for the spontaneous hydrolysis of paraoxon. Enzymatic activity was calculated from the molar extinction coefficient 1310 M^{-1} cm^{-1}. A unit of paraoxonase activity is defined as 1 nmol of 4-nitrophenol formed per min under the above assay conditions.

Atherosclerosis lesion area

Atherosclerosis lesion area in aortic root sinus sections and atherosclerosis lesion area in en face preparations were determined as previously described (8). Macrophage lesion area was determined by immunostaining of aortic root sinus sections using antibodies for the recognition of mouse tissue macrophages and methods for quantifying areas of interest as previously described (9, 10). The number of mice chosen for each experiment was based on power calculations designed to achieve a significance level of 0.05 with a power of 80% using GraphPad STATMate 2.00 (GraphPad Software, San Diego, CA).

Association of L-4F with niclosamide in vitro and determination of the sensitivity to trypsin digestion

Niclosamide is practically insoluble in water (11). However, in the presence of L-4F, the solubility of niclosamide was greatly increased. Since the pH of the stomach is approximately pH 1.0, we sought to determine if L-4F and niclosamide would associate in vitro at pH 1.0. To this end, 5 mg of L-4F alone, 50 mg of niclosamide alone (purity greater than 98%), or 5 mg of L-4F plus 50 mg niclosamide were homogenized in 5 ml of 0.01 N HCl in an all-glass homogenizer yielding a suspension. The mixtures were incubated at 37°C with gentle mixing for 48 h under sterile conditions. After sitting overnight at room temperature, the tubes were centrifuged at 1800 *g*, which removed free niclosamide as a pellet. The 1800 *g* supernatant from the incubation with L-4F plus niclosamide contained niclosamide associated with L-4F, as well as free L-4F, but it contained no free niclosamide. The 1800 *g* supernatant from the incubations with L-4F alone or L-4F plus niclosamide were adjusted to pH 7.0 with 5 mM ammonium bicarbonate, yielding a concentration of L-4F of 133.3 μg/ml. Trypsin was added to the tubes at a concentration of 2.963 μg/ml, yielding a ratio of L-4F to trypsin of 45:1 (mg:mg). The tubes were incubated at 37°C for one h, and the trypsin digestion mixture was sampled at various times. At each time, a sample was removed, and the reaction was stopped by the addition of trifluroacetic acid (TFA). The samples were then injected into a Varian ProStar 210 HPLC system equipped with a ProStar 325 Dual Wavelength UV-Vis detector with the wavelengths set at 215 nM and 280 nm (Varian Inc., Palo Alto, CA). Mobile phases consisted of solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile). Analyses were performed with a reversed-phase C-18 column (Vydac 218TP54, 4.6 × 250 mm, Hesperia, CA) applying a linear gradient of solvent B of 0–100% over 100 min (flow rate: 1 ml/minute).

To determine if niclosamide inhibited the enzymatic activity of trypsin, we incubated 20 μg/ml of TPCK-treated trypsin (Pierce, Rockford, IL) with 0.5 mg/ml (1.15 mM) Nα-Benzoyl-D,L-arginine 4-nitroanilide or 0.25 mg/ml (0.39 mM) Nα-tosyl-gly-pro-lys-4-nitroanalide (Sigma, St. Louis MO) ± 0.1 mg/ml (3 mM) of niclosamide (Sigma) in 200 μl of a pH 8.2 buffer containing 50 mM Tris and 30% ethanol. The ensuing reaction was monitored by taking absorbance readings 405 nM on a SpectraMax 250 microplate spectrophotometer (Molecular Devices, Mountain View, CA) every 30–60 s at room temperature (24°C).

FTIR-ATR

The 1800 *g* supernatant, prepared as described above and containing L-4F associated with niclosamide as well as free L-4F, was centrifuged at 12,000 *g*. The pellet, which contained only L-4F associated with niclosamide but no free L-4F, was analyzed by Fourier transform infrared (FTIR) spectroscopy-attenuated total reflectance (ATR). Infrared spectra were recorded at 25°C using a Bruker Vector 22™ FTIR spectrometer with a DTGS detector, averaged over 256 scans at a gain of 4 and a resolution of 2 cm^{-1}. Peptide samples were prepared by spreading the material onto a 50 × 20 × 2 mm, 45-degree ATR crystal fitted for the Bruker (Pike Technologies) spectrometer (12). The dry sample was hydrated by passing nitrogen gas that had been saturated with 99.996% pure deuterated water (D2O) (Aldrich Chemical, Milwaukee, WI) through the sample chamber for one h prior to measurement. For determination of the infrared spectrum of L-4F in ethanol, the sample was air-dried from a solution of the solvent onto the ATR crystal surface. Then, the sample was carefully covered with ethanol to saturate the peptide with this solvent. FTIR of the L-4F that was tightly associated with niclosamide was determined by spreading of the peptide-niclosamide centrifuge pellet on the ATR crystal surface using a TeflonTM spatula, allowing it to dry, and then rehydrating with D2O vapor for one h before measuring the spectrum of the sample. The spectrum of L-4F was then obtained by digital subtraction of a peptide-free niclosamide paste spread on the ATR as for the peptide-niclosamide sample above and then hydrated with D2O vapor. The proportions of α-helix, β-turn, β-sheet, and "disordered conformations" were determined by area calculations of component peaks of the FTIR spectra using curve-fitting software supplied Galactic Software (GRAMS/AI, version 8.0; Thermo Electron Corp., Waltham, MA). "Disordered" (also called "random" or "unfolded") conformations exhibit high conformational flexibility and frequently occur in small, solvent-exposed peptides (13). The frequency limits for the different structures were α-helix (1662–1645 cm^{-1}), β-sheet (1637–1613 and 1710–1682 cm^{-1}), β-turns (1682–1662 cm^{-1}), and disordered or random (1650–1637 cm^{-1}) (13).

Other methods

HPLC, gel electrophoresis, lipid and lipoprotein and protein determinations, and mass spectrometry were determined by methods described previously (6). Plasma levels of 4F were determined as described previously (6, 14).

Statistical analyses

Statistical analyses were performed by ANOVA or unpaired two-tail *t*-test using GraphPad InStat version 3.05, 32 bit for Windows 95/NT (GraphPad Software, San Diego, CA).

RESULTS

Niclosamide significantly increases the bioactivity of oral L-4F

Administration of L-4F by stomach tube to apoE null mice immediately after administration by stomach tube of niclosamide improved the HDL-inflammatory index in a niclosamide-dose dependent manner (**Fig. 2A**). Similar results were obtained for the LDL-inflammatory index (Fig. 2B). The LDL-inflammatory index was more or less linear

with the dose of niclosamide administered, but that was not the case for the HDL-inflammatory index.

Oral administration of a solution prepared with both niclosamide and L-4F significantly improved the HDL-inflammatory index (**Fig. 3**). However, a solution prepared with niclosamide alone was ineffective. The HDL-inflammatory index values in Figs. 2A and 4 are in agreement with regard to the direction of change. However, the difference in absolute values demonstrates the variability in response that may be due to the different ages of the mice, the different preparation of the compounds, the different presentation of the compounds, the different doses of the compounds, and the inherent variability of such measurements of biologic activity in these mice.

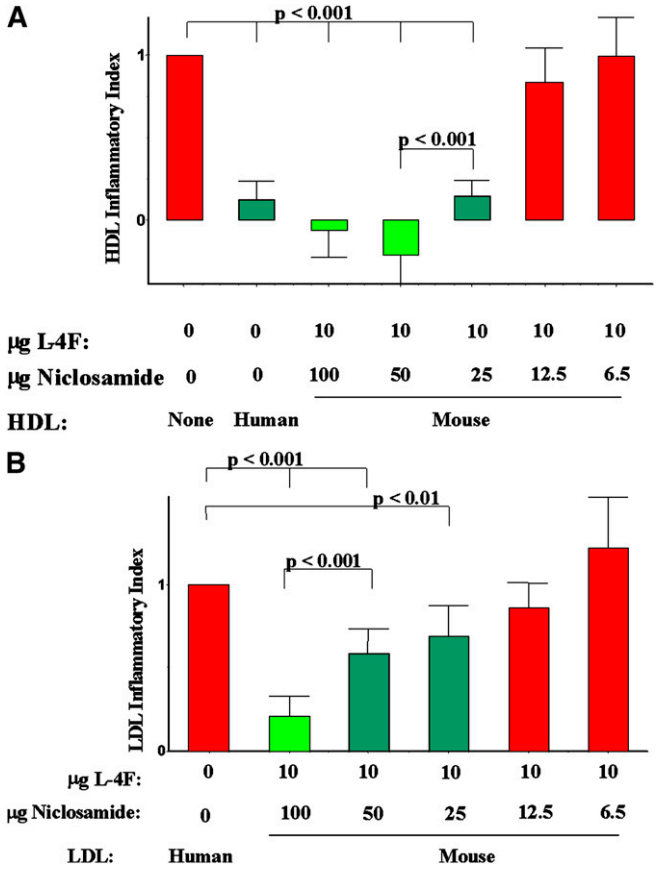

Fig. 2. Administration of L-4F immediately after administration of niclosamide improves the HDL-inflammatory index (A) and the LDL-inflammatory index (B). Twelve-month-old apoE null mice (n = 4 per group) were administered by stomach tube 100 µl water or 100 µl water containing niclosamide in suspension at the dose shown on the X-axis. Immediately after the administration of the first 100 µl, the mice were given a second 100 µl of water containing 10 µg of L-4F. The mice were fasted for 7 h and then bled. A: The HDL-inflammatory index was determined as described in Methods. The first bar on the left is the control human LDL added to the cells in the absence of other additions. This value was normalized to 1.0 as described in Methods. B: The LDL-inflammatory index was determined as described in Methods. The first bar on the left is the control human LDL added to the cells in the absence of other additions. This value was normalized to 1.0 as described in Methods. The data shown are the mean ± SD.

Adding both L-4F and niclosamide to mouse chow or administering niclosamide in the chow and adding the L-4F to the drinking water produced similar improvements in the lipoprotein inflammatory indexes (data not shown).

In the presence of niclosamide D-4F and L-4F have similar oral bioactivities

Oral administration of L-4F together with niclosamide in apoE null mice improved the HDL-inflammatory index (**Fig. 4**) to a degree that was similar to that achieved with administration of D-4F and niclosamide. The increase in paraoxonase activity after administration of L-4F and D-4F with niclosamide was also similar, and the dose response was similar to that observed in Fig. 4 (data not shown). The increased formation of pre–β HDL was similar after oral administration of D-4F without niclosamide compared with L-4F with niclosamide, but the doses required for both L-4F and D-4F for pre–β HDL formation were higher than was the case for the HDL-inflammatory index and paraoxonase activity (data not shown).

Niclosamide increases L-4F plasma levels in apoE null mice

Fasted female apoE null mice 6 months of age (n = 4 per group) were administered by stomach tube ^{14}C-L-4F (21,000 dpm containing 10 µg of L-4F per mouse) with or without 100 µg of niclosamide. Fasting was continued, and the mice were bled nine times over 5–240 min, and the dpm per ml plasma was determined each time. Five minutes after oral administration of ^{14}C-L-4F in the absence of niclosamide, the plasma concentration was 79 ng/ml and

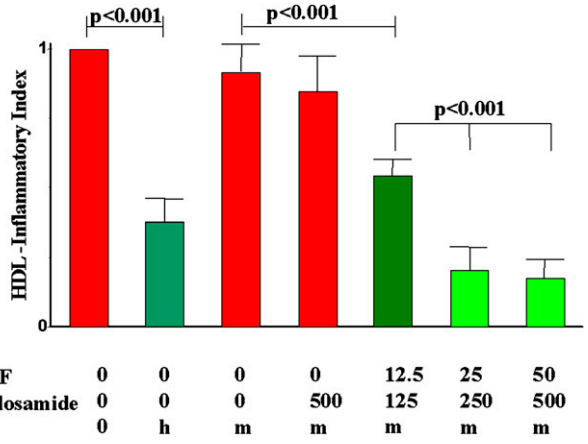

Fig. 3. Niclosamide was added to water at 10 mg/ml or to water containing 1.0 mg/ml of L-4F and was homogenized in a glass-glass homogenizer. The mixtures were stored at 4°C for ten days at which time the supernatants were removed. The supernatant from niclosamide without L-4F contained no niclosamide (i.e., all of the niclosamide had precipitated in the tubes that did not contain L-4F). The solutions were serially diluted and given by stomach tube to seven month-old female apoE null mice (n = 8 per group). Blood was collected 6 h following treatment, and the HDL-inflammatory index was determined as described in Methods. A control normal anti-inflammatory human HDL (h) was included in the assay and all other HDL were from the mice (m). The first bar on the left is the control human LDL added to the cells in the absence of other additions. This value was normalized to 1.0 as described in Methods. The data shown are the mean ± SD.

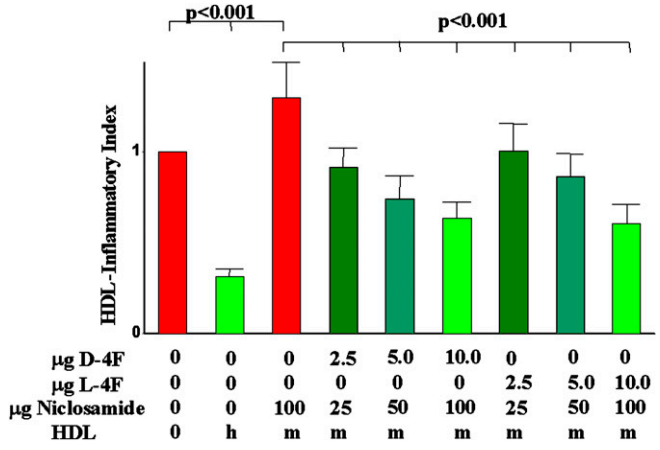

Fig. 4. Oral administration of L-4F together with niclosamide improved the HDL-inflammatory index to a degree that was similar to that achieved with administration of D-4F and niclosamide. Niclosamide was homogenized in a glass-glass homogenizer with or without D-4F or L-4F in a ratio of 10:1 (niclosamide:peptide; wt:wt) in 50 mM ammonium bicarbonate buffer, pH 7.0, containing 0.1 mg/ml Tween-20 (ABCT) and incubated at 37°C for 1 h. The buffer without peptide or with the peptides at the concentrations shown on the X-axis was administered to three-month-old fasting female apoE null mice (n = 8 per group) in a total volume of 100 μl by stomach tube. Six h later the mice were bled, and the HDL-inflammatory index was determined as described in Methods. A control normal anti-inflammatory human HDL (h) was included in the assay and all other HDL were from the mice (m). The first bar on the left is the control human LDL added to the cells in the absence of other additions. This value was normalized to 1.0 as described in Methods.

rapidly declined thereafter. When ^{14}C-L-4F was administered with niclosamide, the maximal plasma concentration 5 min after oral administration was 150 ng/ml, and plasma levels above 100 ng/ml were sustained for another h (data not shown).

Niclosamide increases the oral bioactivity of an apoJ mimetic peptide

4F is a class A amphipathic helical peptide. We have previously published that an apoJ mimetic peptide that is a class G* amphipathic helical peptide when synthesized from all D-amino acids (D-[113-122]apoJ) was orally bioactive in improving HDL inflammatory properties and reducing atherosclerosis in apoE null mice (5). To determine if niclosamide administration would allow this peptide synthesized from L-amino acids to be orally bioactive, the L-amino acid peptide was administered to apoE null mice orally with and without niclosamide. Coadministration of L-[113-122]apoJ and niclosamide improved the HDL-inflammatory index to a degree similar to L-4F given with niclosamide and achieving an HDL-inflammatory index comparable to that obtained with normal human anti-inflammatory HDL (**Fig. 5**).

L-4F plus niclosamide and pravastatin (but not L-4F and pravastatin without niclosamide or niclosamide and pravastatin without L-4F) reduces atherosclerotic lesion area in apoE null mice

Three separate experiments were performed to determine if oral L-4F plus niclosamide would reduce atherosclerotic lesion area in apoE null mice. Since we previously demonstrated that pravastatin and D-4F synergized to improve HDL inflammatory properties and reduce lesions in apoE null mice (9), we included pravastatin in the protocols. The first experiment tested the ability of this oral regimen to prevent lesion formation. The second experiment tested the ability of this regimen to cause lesion regression. The third experiment compared regimens with initial periods of subcutaneously administered peptide to an all-oral regimen. Only the data from the second experiment are shown (**Fig. 6**), but all three experiments were concordant and indicated that oral L-4F with niclosamide and pravastatin is a potent regimen to prevent lesion formation and cause lesion regression in apoE null mice. The data in Fig. 6 indicate that after six months of treatment with L-4F plus niclosamide and pravastatin, the lesion area was actually less than was the case prior to starting treatment. In contrast, the lesion area continued to increase in the mice that only received L-4F and pravastatin without niclosamide or received niclosamide and pravastatin without L-4F.

In the third experiment, subcutaneous L-4F was administered to some of the mice together with oral pravastatin for the first three months during which peak plasma levels of L-4F of 5 to over 45 μg/ml were achieved, compared with plasma levels of approximately 100 ng/ml with the all-oral regimen. The initial period of subcutaneous administration of L-4F did not significantly improve lesions

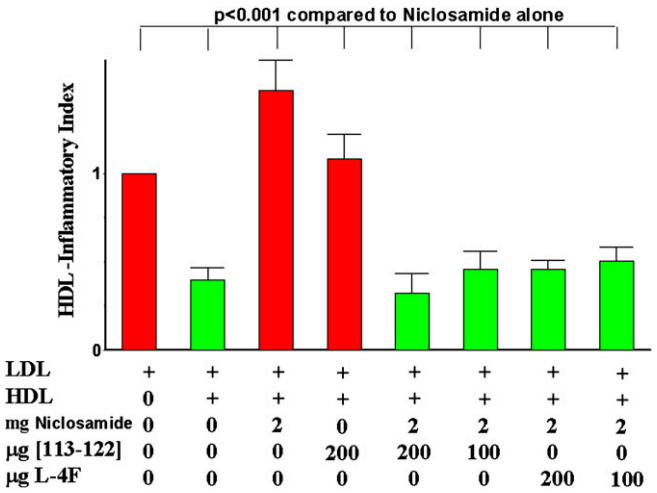

Fig. 5. Oral administration of niclosamide with an apoJ mimetic peptide synthesized from all L-amino acids significantly enhances the ability of the peptide to improve the HDL-inflammatory index of apoE null mice. Ten-month-old apoE null mice (n = 4 per group) were administered by stomach tube 2 mg of niclosamide or 200 μg of L-[113-122]apoJ or 2 mg of niclosamide plus 100 μg or 200 μg of L-[113-122]apoJ or the mice were administered 2 mg of niclosamide plus 100 μg or 200 μg of L-4F. Eight h later the mice were bled, and the HDL-inflammatory index was determined as described in Methods. A control normal anti-inflammatory human HDL was included in the assay and all other HDL were from the mice. The first bar on the left is the control human LDL added to the cells in the absence of other additions. This value was normalized to 1.0 as described in Methods. The data shown are the mean ± SD.

beyond that achieved with the all-oral regimen (data not shown).

L-4F and niclosamide tightly associate in vitro and are orally bioactive

The time of elution in an HPLC system using a C-18 column and a 0–100% acetonitrile gradient in the presence of 0.1% TFA clearly distinguished L-4F from niclosamide (**Fig. 7**). The incubation of niclosamide and L-4F at a ratio of 10:1 (wt:wt) at acid conditions at 37°C resulted in the tight association of L-4F with niclosamide that was easily separated by differential centrifugation from free L-4F and free niclosamide (Fig. 7). Analysis by mass spectrometry did not reveal any evidence of a covalent linkage between L-4F and niclosamide and confirmed that free L-4F and niclosamide that were sham treated were not altered (data not shown). Therefore, the small peaks in Fig. 7C at 33.0 and 39.5 min likely represent products generated during the process of dissolving the 12,000 *g* pellet in TFA. When administered orally, the 12,000 *g* pellet, which contained L-4F tightly associated with niclosamide, was highly effective in improving the HDL-inflammatory index of apoE null mice, whereas neither L-4F alone nor niclosamide alone were effective when administered orally (**Fig. 8**). As previously reported for rabbits (2), subcutaneous injection of L-4F into apoE null mice was also highly effective in improving the HDL-inflammatory index (data not shown). In contrast, subcutaneous injection of niclosamide did not alter the HDL-inflammatory index of the apoE null mice (data not shown), indicating that niclosamide only acts to enhance the oral bioactivity of L-4F but does not influence the HDL-inflammatory index.

The L-4F-niclosamide complex is resistant to trypsin digestion in vitro

Intact L-4F was easily quantified by HPLC (**Fig. 9A**). Incubating L-4F with trypsin in vitro resulted in rapid digestion of the peptide (Figs. 9B, 9C). In contrast, L-4F tightly associated with niclosamide was substantially protected from degradation by trypsin (Figs. 9B, 9C). This protection could have resulted either from steric hindrance or from an inhibitory effect of niclosamide on the enzymatic properties of trypsin. To exclude the latter possibility, we tested trypsin's ability to hydrolyze two chromogenic substrate: Nα-Benzoyl-D,L-arginine 4-nitroanilide (BAPNA) and Na-Tosyl-glycyl-prolyl-lysyl-4 nitroanilide (TGPKNA) in the presence or absence of 3 mM niclosamide. To do so, it was necessary to identify a buffer solution that would support the enzymatic activity of trypsin and keep niclosamide and the substrates in solution. Preliminary experiments showed that 50 mM Tris buffer, pH 8.2 containing 30% v/v ethanol met these criteria. Other preliminary experiments showed that niclosamide was stable in this medium, and that it was not itself a trypsin substrate (data not shown). Fig. 9D shows that even when present in over 2.5-fold molar excess relative to BAPNA or a 7.7-fold excess relative to TGPKNA, niclosamide did not interfere with trypsin's enzymatic activity.

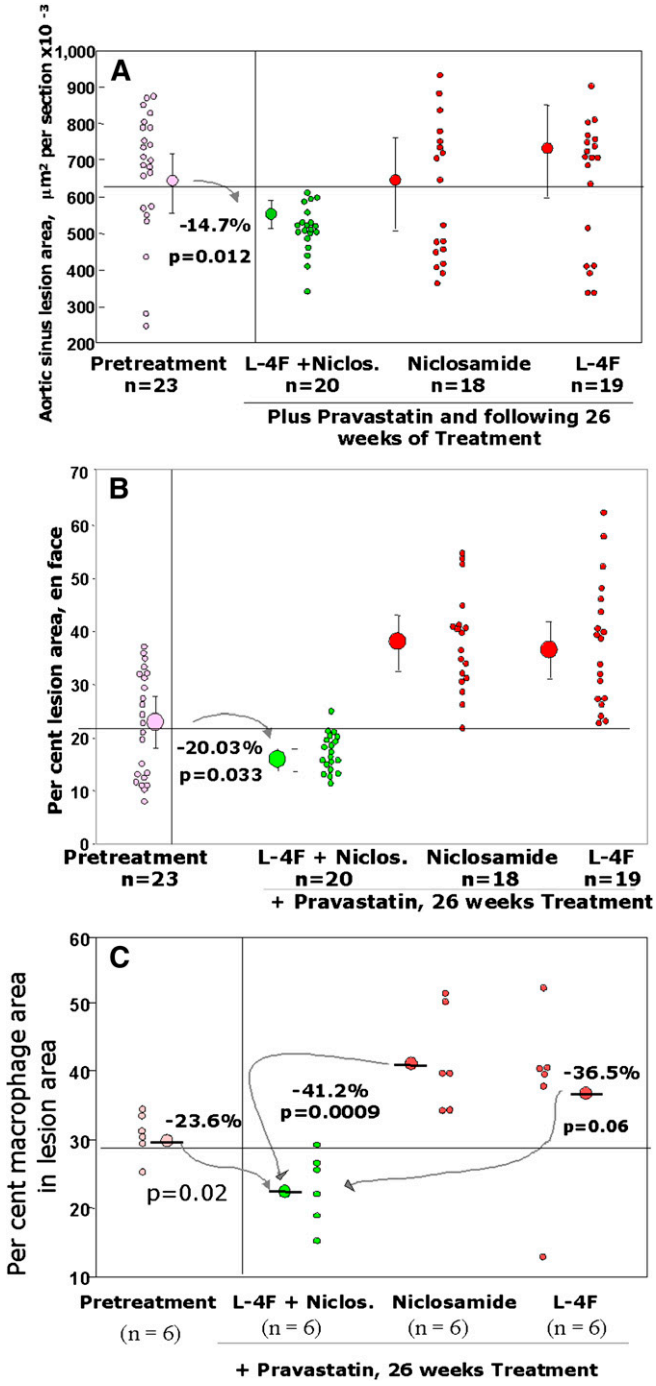

Fig. 6. L-4F plus niclosamide and pravastatin cause lesion regression in old apoE null mice. Female apoE null mice age 9.5 months were divided into four groups. The first group was sacrificed to establish baseline lesion areas. The second group received niclosamide (Niclos.) 2 mg/mouse/day in chow. The third group received L-4F at 200 µg/mouse/day in chow. The fourth group received 2 mg/mouse/day niclosamide plus 200 µg/mouse/day of L-4F in chow. All groups continued past baseline received pravastatin 50 µg/mouse/day in drinking water. After six months the mice were sacrificed and aortic sinus lesion area (A), en face lesion area (B) and macrophage lesion area (C) were determined as described in Methods. The large circles represent the mean ± SD.

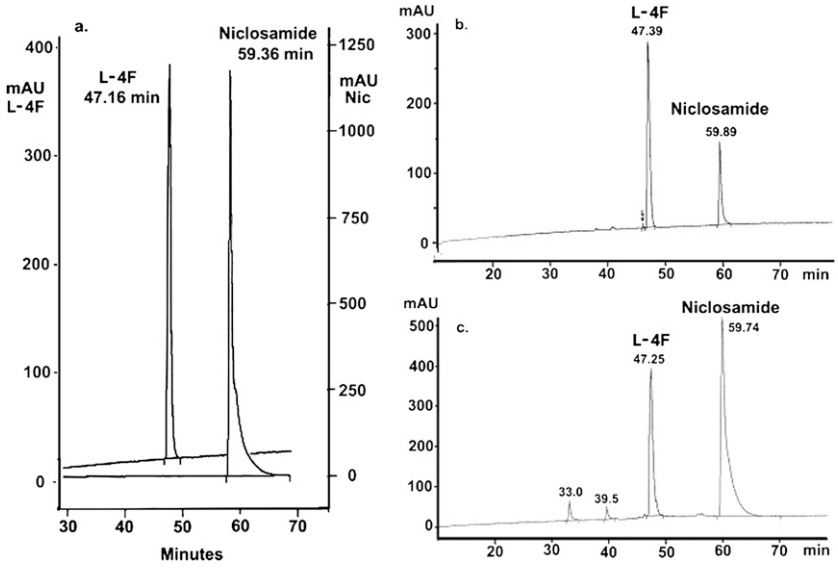

Fig. 7. Chromatography of L-4F alone or niclosamide alone or L-4F tightly associated with niclosamide. Chromatography was performed under standard conditions using a C-18 column and a 1% min^{-1} gradient of acetonitrile in 0.1% trifluoroacetic acid (TFA). A: Niclosamide (100 μg in 100 μl of ethanol) and L-4F (100 μg in 100 μl of buffer) were chromatographed separately under standard conditions. B: Samples were prepared by incubating 50 mg of L-4F in 40 ml of 0.01 N HCl ± 500 mg niclosamide at 37°C for 48 h under sterile conditions. After 48 h, the mixtures were centrifuged for 5 min at 1500 *g*, and the recovered supernatants were centrifuged again for 5 min at 1800 *g*. After storing the resulting supernatants at room temperature overnight, 25 μl was chromatographed under standard conditions. No niclosamide was found in the 1800 *g* supernatant in niclosamide-only incubations (data not shown). C: After overnight storage, the 1800 *g* supernatant was subjected to a 12,000 *g* spin. The pellet that resulted was dissolved in 100 μl of 1% TFA and subjected to HPLC under standard conditions. The supernatant from this spin was also chromatographed and contained only L-4F (data not shown).

FTIR-ATR confirms that niclosamide decreases L-4F self-association

First, the L-4F peptide was examined in ethanol, in which it is freely soluble. FTIR spectra for L-4F in ethanol had a major amide I band centered at 1655 cm^{-1}, indicating a predominant α-helical conformation with only minor contributions from turn and disordered conformations (**Table 1**). When the L-4F was hydrated with D_2O vapor to simulate the peptide in water, there was a decrease in helical conformations and an enhanced β-sheet population, indicated by a signature amide I band at 1630 cm^{-1} and a minor band at 1690 cm^{-1}. As β-sheets require forming multiple intermolecular hydrogen bonds, the occurrence of a sizable β-sheet population suggests either that, at the concentrations used in this study, L-4F underwent a dramatic (and thermodynamically unlikely) intramolecular shape change or that the peptide self-associated and formed molecular aggregates. We favor the second explanation.

When the L-4F peptide was cosolvated with niclosamide and hydrated with D_2O, the helical amide band shifted from 1655 cm^{-1} to 1650 cm^{-1}, indicating that the dominant helical conformation was slightly less ordered (15) and there was a greater representation of random conformations compared with the peptide in ethanol (Table 1). The spectra obtained under the three conditions are shown in **Fig. 10A**. Interestingly there was very little evidence of β-sheet conformations, suggesting that cosolvating the peptide with niclosamide conserved the peptide's helical structure and minimized the formation of β-sheet aggregates. A spectrum of niclosamide alone hydrated with D_2O is shown in Fig. 10B.

These FTIR-ATR studies suggest that L-4F forms a stable helical structure in ethanol similar to the level observed in previous circular dichroism (CD) studies with lipid dispersions of this class A helical peptide (16). At the concentrations of peptide used in the present FTIR study, in the absence of niclosamide there was a sizable β-sheet population in hydrated samples, consistent with the hypothesis that L-4F self-associates in the absence of lipid or some other structure-promoting solvent and consistent with previously published studies using different methods (16). Cosolvating the L-4F peptide with niclosamide minimized the formation of β-sheets and helped conserve the helical structure of the peptide in aqueous environments.

DISCUSSION

Niclosamide is a chlorinated salicylanilide (Fig. 1) that is used as a molluscicide, antihelminthic, and lampricide. Niclosamide has been in clinical use for decades and has shown low toxicity for mammals including humans (4). Niclosamide itself did not inhibit trypsin activity (Fig. 9D). However, L-4F that was tightly associated with niclosamide was highly resistant to trypsin degradation (Figs. 9B, 9C). The interaction of niclosamide with L-4F resulted in less self-association of the peptide in an aqueous environment

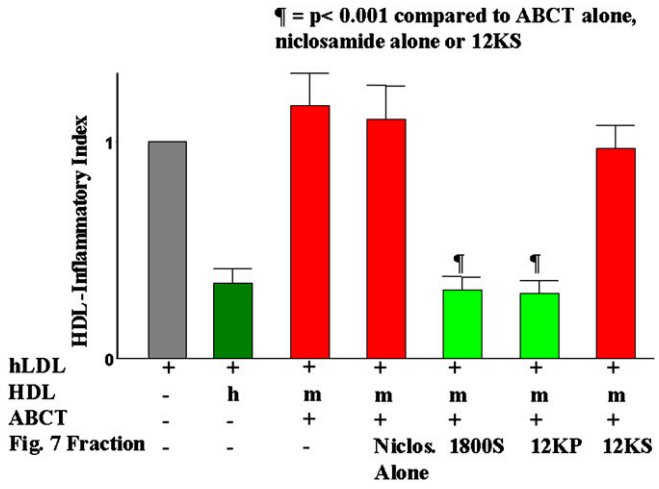

Fig. 8. L-4F that was tightly associated with niclosamide when administered orally significantly improved the HDL-inflammatory index of apoE null mice while neither L-4F alone nor niclosamide alone did. The various fractions from Fig. 7 were administered orally to fasting six-month-old female apoE null mice (n = 4 per group). Vehicle alone (ABCT), 200 μl, or 200 μl ABCT containing 10 μg of niclosamide alone (Niclos. Alone), or 10 μg of L-4F contained in the 1,800 *g* supernatant after incubation of L-4F plus niclosamide (1800S), or 10 μg of L-4F contained in the 12,000 *g* pellet after incubation of L-4F plus niclosamide (12KP), or 10 μg of L-4F contained in the 12,000 *g* supernatant after incubation of L-4F plus niclosamide (12KS) were administered to the mice by stomach tube. Six h later the mice were bled and their lipoproteins fractionated by HPLC, and the HDL-inflammatory index was determined as described in Methods. A control normal anti-inflammatory human HDL (h) was included in the assay and all other HDL were from the mice (m). The first bar on the left is the control human LDL added to the cells in the absence of other additions. This value was normalized to 1.0 as described in Methods. The data shown are the mean ± SD.

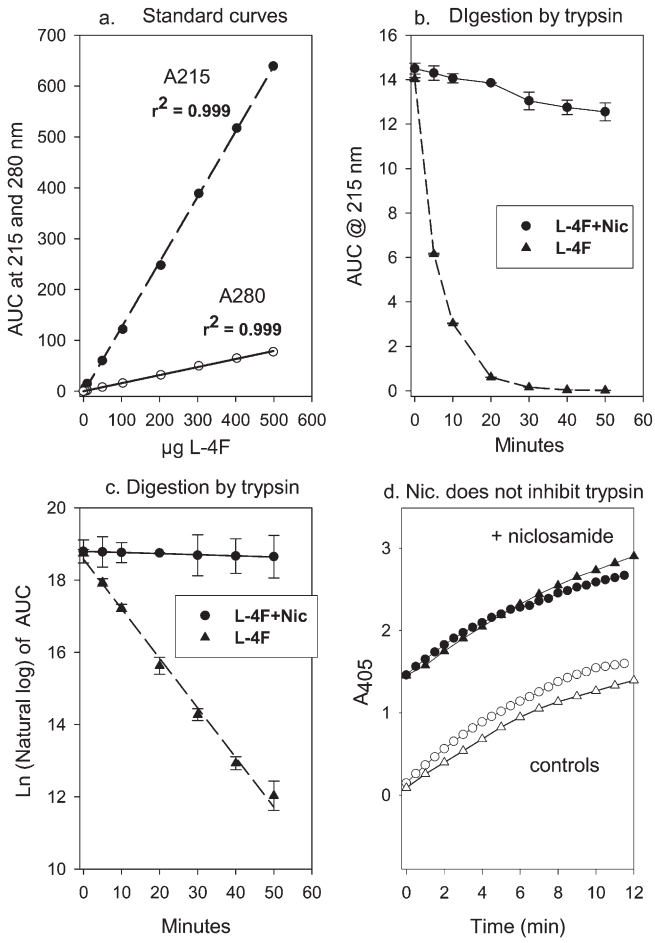

Fig. 9. L-4F tightly associated with niclosamide resists trypsin digestion. A: L-4F was easily quantified by HPLC, as described in Methods. B: L-4F or L-4F tightly associated with niclosamide was incubated with trypsin as described in Methods, and the intact peptide was quantified by HPLC. C: The data shown in (B) were logarithmically transformed. D: The ability of trypsin to digest trypsin-sensitive substrates was determined in the presence and absence of niclosamide. The incubations were done at room temperature in 200 μl of buffer that contained 50 mM Tris, pH 8.2 and 30% v/v ethanol, using 1.15 mM BAPNA (circles) or 0.39 mM TGPKNA (triangles) as chromogenic substrates. Open symbols show substrate hydrolysis in the absence of niclosamide, and solid symbols show it in the presence of 3 mM niclosamide. For BAPNA (Nα-Benzoyl-D,L-arginine 4-nitroanilide), the trypsin was used at 20 μg/ml, and for TGPKNA (Na-Tosyl-glycyl-prolyl-lysyl-4 nitroanilide) trypsin was used at 0.2 μg/ml. Niclosamide has appreciable absorption at 405 nm, and for this reason the "+ niclosamide" curves start higher on the Y-axis. Nic, Niclosamide.

(Table 1 and Fig. 10A). Preliminary molecular modeling suggests that resistance to the action of trypsin maybe due to niclosamide surrounding L-4F in the complex (data not shown), thus protecting the peptide from the action of trypsin, which acts at the C terminus of lysine and arginine residues. The result was a biologic activity after oral administration that was similar to D-4F in a mouse model of atherosclerosis (Fig. 4). In unpublished studies, we have seen that the plasma clearance of L-4F and D-4F are similar as was previously reported by Garber et al. (3) to be the case for the prototypic class A amphipathic helix peptide 18A synthesized from all L- or all D-amino acids. In contrast, the tissue clearance of L-4F and D-4F were quite different, as was the case for the L- and D-peptides reported by Garber et al. (3). L-4F is rapidly degraded once it leaves the circulation, similar to apoA-I (17), whereas D-4F is not (data not shown). Little is known about the binding of niclosamide to peptides and proteins. Zhang and Yap predicted that niclosamide would bind to the SARS-associated coronavirus main proteinase (18), but actual binding studies were not conducted. Since niclosamide is unionized at pH 4 or less (19), one would predict that the association of niclosamide with L-4F in the experiments described in Figs. 7 and 8, in which niclosamide and L-4F were incubated at pH 1.0, was likely not due to charge but to a nonionic interaction of L-4F with niclosamide. The exact nature of this interaction remains to be determined by future studies.

The data presented in this article indicate that it is highly likely that L-4F forms a complex with niclosamide. Since the pH of the stomach is approximately 1.0, we tested the effects of incubating L-4F with niclosamide at this pH. We found that niclosamide alone or L-4F alone was not altered under these conditions. However, when the two were incubated together under these conditions, a tight association resulted, and the L-4F that was associated

TABLE 1. Conformational analysis

	% Conformation			
	α-helix	Turns	β-sheet	Random
L-4F – ethanol	67.26	16.30	0.00	16.44
L-4F – water[a]	34.80	2.20	46.95	16.05
L-4F – Niclosamide[a]	42.43	15.64	18.72	23.21

Mean proportions of secondary structural conformation for L-4F in various structure-promoting environments, as determined by FTIR spectroscopic analysis. FTIR spectra for L-4F tightly associated with niclosamide (L-4F-Niclosamide) were acquired by spreading a peptide-niclosamide paste on the ATR sample surface as detailed in Methods. Tabulated results are mean values from four highly consistent (maximum SEM, 2.4%) measurements. FTIR, Fourier transform infrared spectroscopy.

[a] D_2O-hydrated sample.

with niclosamide could be isolated by differential centrifugation. Centrifugation at 1,800 *g* easily removed free niclosamide. The 1,800 *g* supernatant, following incubation of niclosamide with L-4F, contained both niclosamide and L-4F (Fig. 7). Subjecting this 1,800 *g* supernatant to centrifugation at 12,000 *g* brought down a pellet that contained both L-4F and niclosamide. The 12, 000 *g* supernatant contained only L-4F; there was no niclosamide present. Orally administering the 12,000 *g* pellet to apoE null mice (Fig. 8) reproduced the biologic activity of orally administering L-4F and niclosamide as separate agents given in close temporal proximity (i.e., improved the HDL-inflammatory index as shown in Fig. 2). However, oral administration of the 12,000 *g* supernatant containing only L-4F or oral administration of niclosamide alone was ineffective (Fig. 8). We believe this is compelling evidence that a complex was formed by the interaction of L-4F with niclosamide. However, we have not established the exact molecular weight or stoichiometry of the complex. We have demonstrated by mass spectrometry and analytical HPLC that L-4F tightly associated with niclosamide was unchanged, indicating that there was no covalent modification. Because we have not characterized the complex sufficiently, we have described the interaction between L-4F and niclosamide as "a tight association of L-4F with niclosamide" avoiding the use of the word "complex." Presumably, this "tight association between L-4F and niclosamide" is overcome once the peptide has been absorbed. The oral administration of L-4F with niclosamide improved the HDL-inflammatory index the same as when L-4F was administered by injection without niclosamide, but injection of niclosamide without L-4F did not alter the HDL-inflammatory index. How the "tight association" of L-4F with niclosamide is overcome after absorption to allow the L-4F to interact with cells and lipoproteins, and how the niclosamide is subsequently cleared, are important questions for future studies.

The ability of niclosamide to enhance the biologic activity of an apolipoprotein mimetic peptide after oral administration appears to extend beyond class A amphipathic helical peptides, such as 4F, to at least G* peptides, such as L-[113-122]apoJ (Fig. 5).

The enhanced oral bioavailability of L-4F given with niclosamide was sufficient to prevent and even cause

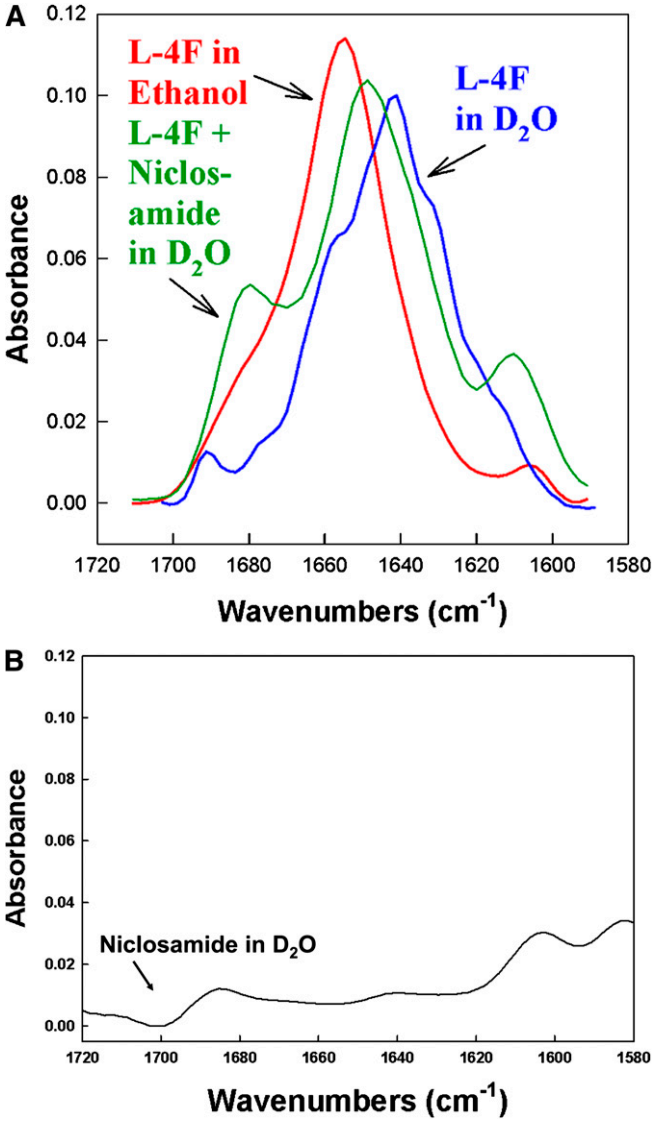

Fig. 10. Cosolvating L-4F with niclosamide minimized the formation of β-sheets and helped conserve the helical structure of the peptide in aqueous environments as determined by Fourier transform infrared spectroscopy (FTIR)-attenuated total reflectance (ATR). A: FTIR-ATR spectra were obtained for samples prepared as described in Methods. B: FTIR spectrum of D2O-hydrated niclosamide paste showing amide I and amide II infrared spectral region. Sample instrumental parameters are the same as those described in Methods.

regression of atherosclerotic lesions in apoE null mice also given pravastatin (Fig. 6). The fact that niclosamide by itself was ineffective in altering the HDL-inflammatory index when given orally (Figs. 2, 8) or when given by subcutaneous injection (data not shown), and the fact niclosamide alone failed to alter atherosclerotic lesions even when given with pravastatin (Fig. 6) strongly suggest that niclosamide acted only to enhance the oral bioactivity of L-4F. The minimal effect of oral L-4F by itself (Fig. 8) or when given with pravastatin without niclosamide (Fig. 6) strongly suggests that apolipoprotein mimetic peptides such as L-4F will need to be given with niclosamide or some other agent that would protect the peptide from proteolytic digestion to be orally effective.

Studies with ApoA-IMilano (20) and recombinant HDL (21) in humans suggest that a good clinical strategy might be an initial period of treatment with apoA-I given parenterally, followed by long-term treatment with an oral statin. In the case of an apoA-I mimetic peptide, it may be that maximal benefit can be achieved by an all-oral regimen as an initial period of treatment with injected L-4F given with oral pravastatin was no better in reducing atherosclerotic lesions in apoE null mice than an all-oral regimen in which L-4F was given orally with niclosamide and pravastatin (data not shown).

The studies reported here suggest that forming a complex between apolipoprotein mimetic peptides synthesized from all L-amino acids and a salicylanilide such as niclosamide maybe an effective method for orally administering these peptides.

M.N., S.H., G.M.A., and A.M.F. are principals in Bruin Pharma, and A.M.F. is an officer in Bruin Pharma.

REFERENCES

1. Navab, M., G. M. Anantharamaiah, S. Hama, D. W. Garber, M. Chaddha, G. Hough, R. Lallone, and A. M. Fogelman. 2002. Oral administration of an apoA-I mimetic peptide synthesized from D-amino acids dramatically reduces atherosclerosis in mice independent of plasma cholesterol. *Circulation.* **105:** 290–292.
2. Van Lenten, B. J., A. C. Wagner, M. Navab, G. M. Anantharamaiah, S. Hama, S. T. Reddy, and A. M. Fogelman. 2007. Lipoprotein inflammatory properties and serum amyloid A levels but not cholesterol levels predict lesion area in cholesterol-fed rabbits. *J. Lipid Res.* **48:** 2344–2353.
3. Garber, D. W., Y. V. Venkatachalapathi, K. B. Gupta, J. Ibdah, M. C. Phillips, J. B. Hazelrig, J. P. Segrest, and G. M. Anantharamaiah. 1992. Turnover of synthetic class A amphipathic peptide analogues of exchangeable apolipoprotein in rats. Correlation with physical properties. *Arterioscler. Thromb. Vasc. Biol.* **12:** 886–894.
4. Botero, D. 1978. Chemotherapy of human intestinal parasitic diseases. *Annu. Rev. Pharmacol. Toxicol.* **18:** 1–15.
5. Navab, M., G. M. Anantharamaiah, S. T. Reddy, B. J. Van Lenten, A. C. Wagner, S. Hama, G. Hough, E. Bachini, D. W. Garber, V. K. Mishra, et al. 2005. An oral apoJ peptide renders HDL anti-inflammatory in mice and monkeys and dramatically reduces atherosclerosis in apolipoprotein E-null mice. *Arterioscler. Thromb. Vasc. Biol.* **25:** 1932–1937.
6. Navab, M., G. M. Anantharamaiah, S. T. Reddy, S. Hama, G. Hough, V. R. Grijalva, A. C. Wagner, J. S. Frank, G. Datta, D. Garber, et al. 2004. Oral D-4F causes formation of pre-β high-density lipoprotein and improves high-density lipoprotein-mediated cholesterol efflux and reverse cholesterol transport from macrophages in apolipoprotein E-null mice. *Circulation.* **109:** 3215–3220.
7. Navab M., S. Y. Hama, G. P. Hough, G. Subbanagounder, S. T. Reddy, and A. M. Fogelman. 2001. A cell-free assay for detecting HDL that is dysfunctional in preventing the formation of or inactivating oxidized phospholipids. *J. Lipid Res.* **42:** 1308–1317.
8. Tangirala, R. K., E. M. Rubin, and W. Palinski. 1995. Quantitation of atherosclerosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL-receptor-deficient and apolipoprotein E-deficient mice. *J. Lipid Res.* **36:** 2320–2328.
9. Navab, M., G. M. Anantharamaiah, S. Hama, G. Hough, S. T. Reddy, J. S. Frank, D. W. Garber, S. Handattu, and A. M. Fogelman. 2005. D-4F and statins synergize to render HDL anti-inflammatory in mice and monkeys and cause lesion regression in old apolipoprotein E-null mice. *Arterioscler. Thromb. Vasc. Biol.* **25:** 1426–1432.
10. Buga, G. M., J. S. Frank, G. A. Mottino, A. Hakhamian, A. Narasimha, A. D. Watson, B. Yekta, M. Navab, S. T. Reddy, G. M. Anantharamaiah, et al. 2008. D-4F reduces EO6 immunoreactivity, SREBP-1c mRNA levels, and renal inflammation in LDL receptor-null mice fed a Western diet. *J. Lipid Res.* **49:** 192–205.
11. Devarakonda, B., R. A. Hill, W. Liebenberg, M. Brits, and M. M. de Villiers. 2005. Comparison of the aqueous solubilization of practically insoluble niclosamide by polyamidoamine (PAMAM) dendrimers and cyclodextrins. *Int. J. Pharm.* **304:** 193–209.
12. Gordon, L. M., K. Y. C. Lee, J. A. Zasadzinski, F. J. Walther, M. A. Sherman, and A. J. Waring. 2000. Conformational mapping of the N-terminal segment of surfactant protein B in lipid using 13C-enhanced Fourier transform infrared spectroscopy. *J. Pept. Res.* **55:** 330–347.
13. Byler, D. M., and H. Susi. 1986. Examination of the secondary structure of protein by deconvolved FTIR spectra. *Biopolymers.* **25:** 469–487.
14. Bloedon, L. T., R. Dunbar, P. Pinell-Salles, R. Norris, B. J. DeGroot, R. Movva, M. Navab, A. M. Fogelman, and D. J. Rader. 2008. Safety, pharmacokinetics, and pharmacodynamics of oral apoA-I mimetic peptide D-4F in high-risk cardiovascular patients. *J. Lipid Res.* **49:** 1344–1352.
15. Tack, B. F., M. V. Sawai, W. R. Kearney, A. D. Robertson, M. A. Sherman, W. Wang, T. Hong, L. M. Boo, H. Wu, A. J. Waring, et al. 2002. SMAP-29 has two LPS-binding sites and a central hinge. *Eur. J. Biochem.* **269:** 1181–1189.
16. Datta, G., M. Chaddha, S. Hama, M. Navab, A. M. Fogelman, D. W. Garber, V. K. Mishra, R. M. Epand, R. F. Epand, S. Lund-Katz, et al. 2001. Effects of increasing hydrophobicity on the physical-chemical and biological properties of a class A amphipathic helical peptide. *J. Lipid Res.* **42:** 1096–1104.
17. Glass, C. K., R. C. Pittman, G. A. Keller, and D. Steinberg. 1983. Tissue sites of degradation of apolipoprotein A-I in the rat. *J. Biol. Chem.* **258:** 7161–7167.
18. Zhang, X. W., and Y. L. Yap. 2004. Old drugs as lead compounds for a new disease? Binding analysis of SARS coronavirus main proteinase with HIV, psychotic and parasite drugs. *Bioorg. Med. Chem.* **12:** 2517–2521.
19. Andrews, P., J. Thyssen, and D. Lorke. 1983. The biology and toxicology of molluscicides, bayluscide. *Pharmacol. Ther.* **19:** 245–295.
20. Nissen, S. E., T. Tsunoda, E. M. Tuzcu, P. Schoenhagen, C. J. Cooper, M. Yasin, G. M. Eaton, M. A. Lauer, W. S. Sheldon, C. L. Grines, et al. 2003. Effect of recombinant apoA-I Milano on coronary atherosclerosis in patients with acute coronary syndromes: a randomized controlled trial. *JAMA.* **290:** 2292–2300.
21. Tardif, J. C., J. Gregoire, P. L. L'Allier, R. Ibrahim, J. Lesperance, T. M. Heinonen, S. Kouz, C. Berry, R. Basser, M. A. Lavoie, et al. 2007. Effect of rHDL on atherosclerosis-safety and efficacy (ERASE) investigators. *JAMA.* **297:** 1675–1682.