This study is to compare the tissue distribution and metabolism of AN1284 after subcutaneous and oral administration at doses causing maximal reductions in IL-6 in plasma and tissues of mice. Anti-inflammatory activity of AN1284 and its metabolites was detected in lipopolysaccharide (LPS) activated RAW 264.7 macrophages. Mice were given AN1284 by injection or gavage, 15 min before LPS. IL-6 protein levels were measured after 4 h. Using a liquid chromatography/mass spectrometry method we developed, we showed that AN1284 is rapidly metabolized to the indole (AN1422), a 7-OH derivative (AN1280) and its glucuronide. AN1422 has weaker anti-inflammatory activity than AN1284 in LPS-activated macrophages and in mice. AN1284 (0.5 mg/kg) caused maximal reductions in IL-6 in the plasma, brain, and liver when injected subcutaneously and after gavage only in the liver. Similar reductions in the plasma and brain required a dose of 2.5 mg/kg, which resulted in 5.5-fold higher hepatic levels than after injection of 0.5 mg/kg, but 7, 11, and 19-fold lower ones in the plasma, brain, and kidneys, respectively. Hepatic concentrations produced by AN1284 were 2.5 mg/kg/day given by subcutaneously implanted mini-pumps that were only 12% of the peak levels seen after acute injection of 0.5 mg/kg. Similar hepatic concentrations were obtained by (1 mg/kg/day), administered in the drinking fluid. These were sufficient to decrease hepatocellular damage and liver triglycerides in previous experiments in diabetic mice. AN1284 can be given orally by a method of continuous release to treat chronic liver disease, and its preferential concentration in the liver should limit any adverse effects.

Keywords Amino-indoline derivative · Interleukin-6 · Liquid chromatography/mass spectrometry · Metabolic oxidation · Tissue distribution

Introduction

An imbalance of pro- and anti-inflammatory cytokines occurs in many pathological conditions. These include among others, rheumatoid arthritis (Feldmann et al. 1996; Costa et al. 2018), ulcerative colitis (Strober and Fuss 2011), type 2 diabetes (Rehman and Akash 2016), acute liver failure (Granger and Remick 2005), acute lung disease (Dolinay et al. 2012), and non-alcoholic fatty liver disease (NAFLD) (Farrell et al. 2018). Most of the orally administered drugs used to treat these conditions do not provide adequate relief (Miossec 2014; Golabi et al. 2017; Damiao et al. 2019), and currently, no drug is effective for the treatment of NAFLD (Boeckmans et al. 2019). Although monoclonal antibodies or soluble receptors, given to block TNF-α, are very effective in treating rheumatoid arthritis and ulcerative colitis, they have to be administered parenterally (Schwartzman and Morgan 2004) and are very expensive (Ollendorf et al. 2009). Moreover, they can increase the risk of tuberculosis and demyelination disorders (Khanna and Feagan
cutaneously implanted mini-pumps, which was effective in a similar amount of drug in the liver to that given by sub-
given chronically in the drinking fluid and would produce the wanted to find the concentration of AN1284 that could be
plasma and tissues and use it to compare drug distribution sensitive enough to detect AN1284 and any metabolites in
liquid-chromatography mass spectral (LC–MS/MS) method for the prediction of the therapeutic outcome and any poten-
tial adverse effects. Thus, the second aim was to develop a
tial Location of AN1284 and other indoline derivatives
in previous experiments (Finkin-Groner et al. 2015, 2017;
Shifrin et al. 2017).

As oral administration is more convenient than injec-
tions and has better patient compliance, the first aim of this study was to find the dose of AN1284 that would cause the maximum reduction in plasma and tissue cytokines in LPS-treated mice when given by gavage like that seen after sc injection. Previously, it was shown that AN1284 is rapidly degraded by both mouse and human microsomes (Maimon 2016, Suppl. S1). Knowledge of the metabolism and phar-
macokinetics of a drug intended for clinical use is essential for the prediction of the therapeutic outcome and any poten-
tial adverse effects. Thus, the second aim was to develop a
liquid-chromatography mass spectral (LC–MS/MS) method sensitive enough to detect AN1284 and any metabolites in
plasma and tissues and use it to compare drug distribution and metabolism after sc and oral administration. Lastly, we
wanted to find the concentration of AN1284 that could be given chronically in the drinking fluid and would produce a similar amount of drug in the liver to that given by sub-
cutaneously implanted mini-pumps, which was effective in reducing liver damage.

Materials and methods

Animals

All experimental procedures in mice complied with the Principles of Laboratory Animal Care (NIH publication #85–23, revised 1985) and were performed according to protocols approved by the Ethics Committee of the Hebrew University. Male ICR mice, 7–8 weeks old, provided by Envigo (Jerusalem, Israel), were used for these experiments. Mice were housed in groups of 5 per cage in a pathogen-free unit under controlled 12-h light/12-h dark cycle (lights on at 7:00 and lights off at 19:00) and an ambient temperature of 21 ± 1 °C and 40–50% humidity, with free access to standard rodent chow (Envigo, Israel) and water. The mice were acclimatized to the animal facility for at least 5 days before the experiment.

Compounds and reagents

The compounds used for the study were, AN1284 (Molecular weight MW 291.26 g/mol, free base 218.34 g/mol) and AN1422 (3-(indole-1-yl)-N-isopropylpropan-1-amine (Zeeli et al. 2018), (its oxidized metabolite, MW 216.33 g/mol) and the 4, 5 and 7-OH metabolites of AN1284 (MW 234.34 g/mol). LPS (from Escherichia coli, serotype (0111:B4). Riv-
astigmine hemitartrate was a gift from Novartis, Basle, Swit-
zeerland. LC–MS/MS grade methanol, ultra-high pressure liquid chromatography (UHPLC) grade water, and acetoni-
trile (MeCN) were purchased from Biolab Ltd., Israel. GHP membranes were purchased from Pall Corporation, NY, USA, and formic acid from Merck (Darmstadt, Germany).

Measurement of nitric oxide and cytokines in LPS activated RAW 264.7 cells

The experiments were carried out as described in Zeeli et al. 2018, with minor modifications. RAW 264.7 cells (Euro-
pean Collection of Authenticated Cell Cultures (ECACC, passages 4–5)) were seeded at a density of 50×10³ cells per well in 96-well culture plates. The steroid, budeson-
ide was used as a positive control. AN1284 or budesonide was added to the cells and medium containing 2% fetal calf serum to give final concentrations ranging from 1×10⁻³ to 1×10⁻¹⁸ M. Once the indole metabolite, (codenamed AN1422) and the 7-OH metabolite of AN1284 (codenamed AN1280) were identified, they were synthesized and added to RAW 264.7 cells at similar concentrations to those of AN1284. The cells were incubated for 2 h at 37 °C prior to stimulation with LPS (2.5 μg/mL). After 8 h, supernatants were harvested for measurement of TNF-α and after 24 h for
IL-6 and nitric oxide (NO) as previously described (Zeeli et al. 2018). At least two experiments using four or six replications of each concentration were performed.

**Measurement of IL-6 in plasma and tissues**

In previous studies, we showed that IL-6 protein could be reliably measured in the plasma, brain, and liver, 4 h after intraperitoneal (ip) injection of LPS (Finkin-Groner et al. 2015). We also found that the reduction of IL-6 by AN1284 in the plasma was greater when the drug was given 15 min before LPS than after 30 min. Mice were randomly distributed into seven groups and injected subcutaneously either with saline, 1 mL/100 g, or AN1284 (10–21 mice per dose) (0.05, 0.5 mg/kg or 2.5 mg/kg), dissolved in saline, or AN1422, dissolved in DMSO, diluted 100-fold in saline, at doses of 0.44 or 2.2 mg/kg (8–15 mice per dose), or DMSO diluted 100-fold as vehicle control, 15 min before ip injection of LPS (5 mg/kg). Other groups of 7–16 mice were given water (10 mL/kg) or AN1284 (0.5, 2.5, or 10 mg/kg) by gavage, all 15 min before LPS. Four hours later, the mice were deeply anesthetized with ketamine 100 mg/xylazine, 10 mg/kg, and blood collected by cardiac puncture. The liver, brain, and kidneys were rapidly removed, snap-frozen in liquid nitrogen, and stored at -80 °C until assayed for IL-6 as previously described (Finkin-Groner et al. 2015).

**Measurement of AN1284 and AN1422 in plasma and tissues**

Oral administration of AN1284 (2.5 mg/kg, 2.2 mg/kg base) produced a maximal reduction in IL-6 in plasma of LPS injected mice, similar to that seen after 0.5 mg/kg (0.44 mg/kg base) injected sc. Therefore, these doses were used to measure the concentrations of AN1284 and its metabolites in the plasma and tissues, and four mice were given saline to serve as controls. After drug administration by the oral or sc route, the mice were deeply anesthetized with ketamine (100 mg/kg) + xylazine (10 mg/kg) and decapitated 5, 10, 20, 30, 45, or 60 min later. Blood was collected into tubes containing 10 µL EDTA (0.5 M); the liver, brain, and kidneys were rapidly removed, snap-frozen in liquid nitrogen, and stored at -80 °C.

Since chronic sc administration of AN1284 by minipumps at concentrations of 1 and 2.5 mg/kg/24 h significantly reduced signs of liver damage in mice on a high fat diet (unpublished observations) or in db/db mice with type 2 diabetes (Perymyakova et al. 2020), we also gave these doses and method of administration to two groups of 8 mice and measured the concentration of AN1284 and the indole metabolite in the liver. After we found that oral and sc administration of AN1284 (0.5 mg/kg) caused a similar reduction in liver cytokines, we gave the drug in the drinking water to groups of 8 mice to provide concentrations of 0.25, 0.5 or 1 mg/kg/day for 48 h and measured the levels of drug in the liver.

The urinary excretion of AN1284 and any metabolites was measured in seven mice, injected sc with AN1284 (0.5 mg/kg) or given (2.5 mg/kg) by gavage that were housed in individual metabolism cages. Urine was collected for 24 h, passed through 0.45-μm filters. The pH was measured, and the samples were stored at -80 °C until analyzed by LC–MS/MS.

Samples collected from all mice given AN1284 or saline were prepared for LC–MS/MS analysis as follows. To 160 µL of plasma, 20 µL of internal standard solution (rivastigmine 750 ng/mL) and 20 µL of ultra-pure water were added. The mixture was vortexed for 5 min and then allowed to stand for a further 5 min, and the procedure repeated three times. To precipitate proteins, 300 µL of ice-cold HPLC grade MeOH was added to each sample which was vortexed for 10 min and allowed to stand for another 10 min and also repeated three times. The samples were centrifuged at 20,800 g at 4 °C for 15 min; the supernatants were withdrawn into an Eppendorf tube; and 200 µL was filtered through 0.25-μm GHF membranes and stored at -80 °C until injection into the LC–MS/MS instrument. Samples from the brain and liver were prepared as described for plasma with minor modifications. The tissues were homogenized (100 mg/mL, Polytron, Kinematica GmbH, Germany) in phosphate buffered saline. Twenty µL of internal standard was added to 180 µL of tissue homogenate, followed by MeOH and treated as described for plasma. Calibration curves for different concentrations of AN1284 and AN1422 added to the plasma, brain, kidney, or liver homogenates prepared from control mice were also run for each experiment. The equation (y = a + bx) for each curve was obtained by linear least-squares regression of the measured peak area (y) versus the concentration added to the biological matrix (x), corrected for the peak area of the internal standard rivastigmine, added to each sample. After measurement of the volume and pH, 200 µL of urine samples were stored in vials at -80 °C until injection into the LC–MS/MS instrument. The area under the curve (AUC) of the concentration of AN1284 and AN1422 in plasma and tissues as a function of time was calculated after oral and sc administration of AN1284.

**Analysis of plasma, tissue, and urine samples**

LC–MS/MS provides a powerful tool for the detection and quantification of specific analytes in mixtures from biological extracts that contain a number of compounds. Since many compounds may share the same exact mass, a combination of the following parameters distinguishes the desired component from analogs in the extract: (1) retention time of the compound on the column; (2) exact mass of the molecule.
**a**

Counts (%) vs Acquisition time (min)

**b**

Counts (%) vs Mass to Charge (m/z)

**c**

AN1284 glucuronide
Urine samples were injected into the LC–MS/MS machine at a voltage of 4.5 kV. Standards were prepared at a concentration of 1 mg/mL in methanol.

The temperature was set to 350 °C, and the ion spray voltage was adjusted. The ESI was operated in the positive mode. The source parameters were readjusted to account for scan mode and collision energy for product ion mode. Specific parameters of the ion source were readjusted by varying the fragmentor voltage of the ion source.

In samples prepared from mice given AN1284, a small increase in the level of the total ion chromatogram, that contains the information of all the ions found in the sample, was observed at a retention time close to that of AN1284 (Fig. 1a). The MH+ ion of AN1284 is 219.185, and the MS spectrum of that zone in the chromatogram revealed an increase in the mass of 217.169 that displayed Gaussian behavior. This was not observed in solutions of AN1284 unexposed to biological media. Calculations of the difference between parent ions for the drug AN1284, 219.185 [MH+] and its metabolite 217.169 [MH+] corresponded to the indole with an exact mass of two fewer hydrogens. To confirm the identity of this metabolite, we compared the mass spectral parameters, retention time, ion parent mass, and daughter ion peaks of AN1284 to that of the indole AN1422 (Zeeli et al. 2018). AN1284 and AN1422 share a common product ion of 132.080, indicating that the loss of the 2 hydrogens also occurs under the conditions of the collision cell-electric voltage and nitrogen bombardment. Although AN1284 contains two compounds were identical. However, we found that the position of the OH group dramatically influenced the retention time of the compounds on a LC column. Therefore, we injected urine from control mice to which was added amounts of the individual compounds into the LC–MS/MS apparatus as described for the analysis of the metabolites found in urine after administration of AN1284 to the mice. We found that only the 7-OH substituted derivative (AN1280) had the same retention time as the metabolite found in the urine of drug treated mice, confirming the position of metabolic oxidation.

**Statistical analysis**

All doses and concentrations of AN1284 and AN1280 are expressed in terms of its 2HCl salt and AN1422 as a free base, and the results are presented as the mean ± STD. Differences in the % of LPS control in NO, TNF-α, and IL-6 released from RAW 264.7 cells produced by similar concentrations of budesonide, AN1284, AN1422, and AN1280 and % of levels of IL-6 after saline and LPS induced by different doses of AN1284 administered to mice, were analyzed by ANOVA using IBM SPSS Statistics Version 25 followed by Duncan’s post hoc test. A p value of < 0.05 was considered to be significant.

**Results**

**Metabolism of AN1284**

In samples prepared from mice given AN1284, a small increase in the level of the total ion chromatogram, that contains the information of all the ions found in the sample, was observed at a retention time close to that of AN1284 (Fig. 1a). The MH+ ion of AN1284 is 219.185, and the MS spectrum of that zone in the chromatogram revealed an increase in the mass of 217.169 that displayed Gaussian behavior. This was not observed in solutions of AN1284 unexposed to biological media. Calculations of the difference between parent ions for the drug AN1284, 219.185 [MH+] and its metabolite 217.169 [MH+] corresponded to the indole with an exact mass of two fewer hydrogens. To confirm the identity of this metabolite, we compared the mass spectral parameters, retention time, ion parent mass, and daughter ion peaks of AN1284 to that of the indole AN1422 (Zeeli et al. 2018). AN1284 and AN1422 share a common product ion of 132.080, indicating that the loss of the 2 hydrogens also occurs under the conditions of the collision cell-electric voltage and nitrogen bombardment. Although AN1284 contains two
amine moieties and AN1422, only one, a comparison of their ion efficiencies, revealed that AN1422 ionizes five times better than AN1284. Therefore, although the levels of AN1284 in the plasma and tissues are higher than those of AN1422, the areas under the curve of the peaks for the same concentrations of AN1284 are about fivefold lower.

**Reduction of NO and cytokines by AN1284 and AN1422 in LPS-activated RAW 264.7 macrophages**

The increase in NO and cytokines in response to LPS in macrophages in the different experiments ranged from 1.0 to 3.7 μM for NO; from 1.0 to 4.5 nM for TNF-α; and from 2.8 to 17.8 nM for IL-6. AN1284 (0.1 pM) significantly reduced NO, TNF-α, and IL-6, \( p < 0.01 \). A maximal reduction of ≈ 40% in NO and cytokines was seen at a concentration of 1 pM, and no further reductions were achieved by increasing the concentration as high as 10 nM. Budesonide had a similar effect on NO and cytokines to that of AN1284 at almost all concentrations except 10 nM (Fig. 2). AN1422 caused a smaller reduction (20–24%) than AN1284 in NO and cytokines at all but the lowest concentration. AN1280 was also less effective than AN1284 in reducing NO and TNF-α, but not IL-6 (Fig. 2).

**Reduction by AN1284 and AN1422 of IL-6 in plasma and tissues of LPS injected mice**

In control mice given saline, the concentrations of IL-6 in the plasma, brain, and liver, 4 h after ip injection of LPS, were 7.41 ± 0.12 ng/mL, 0.50 ± 0.04 ng/gm, and 0.60 ± 0.04 ng/gm, respectively. AN1284 (0.5 mg/kg) injected 15 min before LPS, which caused maximal reductions in IL-6 in the plasma, brain, and liver of 31, 38, and 36%, respectively, while AN1422 (0.44 and 2.2 mg/kg) reduced IL-6 significantly only in the plasma (Table 1). When given orally, AN1284 (0.5 mg/kg) reduced IL-6 in the liver by 34.6%, but only by 17.7 and 19.9%, respectively, in the plasma and brain. A dose of 2.5 mg/kg caused a similar reduction in IL-6 in the liver to that of 0.5 mg/kg, but 10 mg/kg, was no longer effective. However, 2.5 mg/kg was needed to cause a significant decrease in the plasma and brain to those seen after sc injection of 0.5 mg/kg.

Previously, we had shown that sc injection of AN1284 reduced TNF-α in the plasma of LPS injected mice (Zeeli et al. 2018). Because of the important role played by TNF-α in inflammatory disease, we measured the cytokine in the plasma samples collected after oral administration of AN1284. At a dose of 2.5 mg/kg, AN1284 decreased plasma levels of TNF-α by 41.4 ± 18.4%.

**Concentrations of AN1284 and AN1422 in plasma and tissues**

To compare plasma and tissue distribution of AN1284 and any metabolites after sc and oral administration, we used doses of 0.5 mg/kg and 2.5 mg/kg, respectively, because they caused similar maximal reductions in IL-6 in plasma and tissues. The lowest detectable amount of AN1284 was 1.25 ng/mL and of AN1422, 0.125 ng/mL. The concentrations time curves of AN1284 and its metabolite in plasma and tissues after administration of these doses of AN1284 are shown in Fig. 3a and b. Within 5 min of sc injection, peak levels
of AN1284 were seen in the plasma and kidneys and after 10 min in the brain and liver.

AN1284 was also rapidly absorbed after oral administration, but the relative distribution in plasma and tissues and the amounts of drug and metabolite differed substantially from those after sc injection. Thus, drug levels peaked at 5 min in the liver and only after 10 min in the plasma, brain, and kidneys. The area under the plasma concentration vs.

time from administration curve from time zero to the time (t) (60 min) of the last quantifiable plasma concentration, AUC, for AN1284, calculated by means of the log/linear trapezoidal rule from pre-dose concentrations to t, was 83.54 ng/h/mL for sc injection of 0.5 mg/kg and 9.91 ng/h/mL for oral administration of 2.5 mg/kg. The time course of the indole metabolite, AN1422, generally followed those of AN1284 (Fig. 3a and b). The peak levels of AN1284 and AN1422 and AUC for each compound in plasma and tissues after sc and oral administration, together with the ratios of the values for AN1422 to those of AN1284, are shown in Table 2. With the exception of the liver, all the ratios were higher after oral than after sc administration, but in the brain, both the peak concentration and AUC of the metabolite were almost identical to those of AN1284.

The concentrations of AN1284 and AN1422 in the liver of mice given two doses of the drug chronically via sc implanted mini-pumps, or three doses in the drinking fluid, are shown in Table 3. These were similar after 2.5 mg/kg/day administered via sc mini-pumps and 1 mg/kg/day given in the drinking fluid. However, as might be expected, there was more inter-mouse variability when given by the latter route.

Cumulative 24-h excretion of AN1284 and metabolites in urine

Despite the relatively high concentrations of AN1284 in the kidneys after sc injection, the 24-h urinary levels were only 0.38% of the administered dose (Table 4). They were even smaller (about 0.007%) after oral administration of a fivefold higher dose, in keeping with the much lower concentrations of AN1284 found in the kidneys when given by this route. The low excretion of the compound could be explained by the urinary pH of 7.0–7.5 that would favor reabsorption of the basic drug. To discover any hydrophilic metabolites of AN1284 that would be more readily excreted, we scanned the urine samples and found two more drug-related spe

Table 1 Percent reduction by AN1284 and AN1422 of LPS-induced elevation of IL-6 in plasma and tissues

| Dose (mg/kg) | Plasma | Brain | Liver |
|-------------|--------|-------|-------|
| Saline      | 1 mL/kg| ±11.4(28) | ±16.8(27) | ±16.8(27) |
| AN1284      | 0.05   | 10.8±19.3 (10)b | 22.3±19.3 (11)** | 8.9±25.3 (11)c |
|             | 0.5    | 31.4±15.6 (15)** | 37.8±17.4 (13)** | 35.6±16.1 (15)** |
|             | 2.5    | 25.0±26.0 (15)** | 7.9±14.1 (11)b | 31.4±15.8 (10)** |
| Vehicle     | 1 mL/kg| ±15.7 (12) | ±6.0 (6)  | ±18.4 (8)  |
| AN1422      | 0.44   | 11.4±18.6 (11)d | 9.5±21.9 (9) | 21.8±16.8 (8) |
|             | 2.2    | 26.2±17.8 (15)** | 17.9±18.1 (8) | 20.2±17.7 (8) |

Data represent mean % reduction±STD in IL-6 in mice injected with LPS 5 mg/kg ip, induced by drug treatment compared to value in saline or vehicle pretreated controls. () number of mice

ANOVA: AN1284 sc. Plasma F(3, 42) = 7.08; p < 0.01. Significantly different from AN1422 (2.2 mg/kg) sc, d

ANOVA: AN1284 gavage. Plasma F(3, 42) = 7.86; p = 0.0001. Brain F(3, 49) = 9.54; p = 0.0001. Liver F(3, 58) = 13.33; p < 0.0001. AN1422 sc. Plasma F(2, 20) = 2.21; p = 0.05

| Dose (mg/kg) | Plasma | Brain | Liver |
|-------------|--------|-------|-------|
| AN1284      | 0.5    | 17.7±20.9 (11)* | 19.9±17.2 (8) = | 34.6±17.3 (11)** |
|             | 2.5    | 37.6±19.0 (13)** | 36.2±19.9 (16)** | 28.1±14.3 (12)** |
|             | 10     | 15.7±16.9 (11)* | 19.3±17.3 (13)** | 3.9±18.1 (11)d |

Significantly different from saline or vehicle, *p < 0.05; **p < 0.01; significantly different from AN1422 (0.5 mg/kg) sc, *p < 0.05, **p < 0.01; significantly different from both AN1284 (0.5 mg/kg) and (2.5 mg/kg) sc, *p < 0.01. Significantly different from AN1422 (2.2 mg/kg) sc, *p < 0.05. Significantly different from AN1424 (2.5 mg/kg) gavage, *p < 0.05; **p < 0.01; significantly different from AN1284 (0.5 mg/kg) and (2.5 mg/kg) gavage, */p < 0.01.
Fig. 3  Concentration–time curves of AN1284 and AN1422 in plasma and tissues. a After sc injection. b After gavage. Data represent the mean ± STD from 7 to 16 mice per time point.
the peak areas of the glucuronide to those of AN1280, we estimated that there was probably twice as much after sc injection of AN1284 and almost 15 times as much of the glucuronide after oral administration (Table 4).

The smallest amount of AN1280 that was detectable in solution was 4.6 ng/mL (equivalent to a tissue concentration of 52 ng/gm). Five and ten minutes after oral administration of AN1284, the concentrations of AN1280 in the livers of three of the seven mice in which we were able to measure it were 692 ± 45 ng/gm and 410 ± 17 ng/gm, respectively, but were below the limits of detection in plasma and kidneys. By contrast, it was possible to measure the 7-OH glucuronide in the liver, plasma, and kidneys in all the mice at both time points. Estimation of the amounts of the glucuronide from a comparison of the peak areas relative to those of the 7-OH derivative showed them to be about 50 times higher in the liver, 5 and 10 min after drug administration. The estimated concentrations of

Table 2 Peak concentrations and AUC of AN1284 and its indole metabolite, AN1422, in plasma and tissues after subcutaneous and oral administration

| Tissue          | AN1284   | Tissue/plasma | AN1422   | AUC (ng x h/mL) | AUC (ng x h/mL) | AN1422/AN1284 | Peak conc |
|-----------------|----------|---------------|----------|-----------------|-----------------|---------------|-----------|
|                  | Cmax a (ng/gm or mL) |                  | Cmax a (ng/gm or mL) |                |                |              |           |
| **Subcutaneous administration (0.5 mg/kg)** |          |               |          |                |                |              |           |
| Plasma           | 184 ± 80 | 83.5          | 64.2 ± 29.7 | 33.3           | 0.35            | 0.40          |           |
| Brain            | 1070 ± 319 | 5.8 (5.2–7.1) | 323 ± 92  | 300            | 0.30            | 0.41          |           |
| Liver            | 285 ± 140 | 1.6 (1.5–1.8) | 114 ± 66.2 | 65.6           | 0.40            | 0.35          |           |
| Kidneys          | 3733 ± 1182 | 20.3 (18.6–24.5) | 1457 ± 318 | 730           | 0.39            | 0.47          |           |
| **Oral administration (2.5 mg/kg)** |          |               |          |                |                |              |           |
| Plasma           | 32.8 ± 14.9 | 9.91          | 18.3 ± 10.6 | 5.47           | 0.57            | 0.55          |           |
| Brain            | 65.2 ± 21.0 | 2.0 (1.8–2.3) | 62.4 ± 26.3 | 36.9           | 0.96            | 0.88          |           |
| Liver            | 1553 ± 519 | 47.3 (43.4–57.8) | 558 ± 174 | 207           | 0.36            | 0.49          |           |
| Kidneys          | 180 ± 71 | 5.5 (5.3–6.1) | 114 ± 44  | 76.5           | 0.63            | 0.64          |           |

AUC area under curve of concentration of AN1284 or AN1422 in plasma or tissues over time. aValues represent the mean ± STD of 6–11 replicates per tissue or plasma. bRatio of peak concentrations in tissue/peak concentrations in plasma. cRatio of AUC in tissue/AUC in plasma.

Table 3 Concentrations in the liver of AN1284 and its indole metabolite (AN1422) after chronic administration of AN1284 to mice by sc implanted mini-pumps or in the drinking fluid

| Dose Route          | Route | A. Conc AN1284 | B. Conc AN1422 | Ratio A/B |
|---------------------|-------|----------------|----------------|-----------|
| 0.5 mg/kg sc        |       | 285            | 114            | 2.5       |
| 2.5 mg/kg oral      |       | 1553           | 523            | 3.0       |
| 1 mg/kg/day sc (mps)|       | 26.4 ± 3.1     | 7.8 ± 3.3      | 3.4       |
| 2.5 mg/kg/day sc (mps)|       | 36.2 ± 7.3    | 13.7 ± 3.6    | 2.6       |
| 0.25 mg/kg/day Drinking water | 15.3 ± 6.0 | 4.2 ± 3.7 | 3.6       |
| 0.5 mg/kg/day Drinking water | 26.3 ± 12.5 | 7.5 ± 4.9 | 3.5       |
| 1 mg/kg/day Drinking water | 36.4 ± 12.3 | 11.8 ± 6.7 | 3.2       |

mps Mini-pumps

Table 4 Urinary excretion of AN1284 and its metabolites after subcutaneous and oral administration

| Route | Average weight (g) | Dose of AN1284 (µg base/mouse) | Concentration in urine (ng/24 h) | Ratio peak areas |
|-------|--------------------|---------------------------------|----------------------------------|-----------------|
|       |                    | AN1284                          | AN1422                          | AN1280          | Glucuronide/AN1280 |
| Sc (7) | 28.6 ± 2.3         | 12.6 ± 1.0                      | 47.4 ± 27.2                     | 31.4 ± 23.3     | 320 ± 177 2.2 ± 1.9 |
| Oral (7) | 29.7 ± 2.5       | 63.8 ± 1.7                      | 4.7 ± 0.6                       | 62.7 ± 14.8     | 774 ± 443 9.5 ± 7.7 |

Data represent mean ± STD or 24-h urine collections; (number of mice); AN1422 is the indole and AN1280, the 7-OH derivative of AN1284.
Discussion

In all our previous studies, the indoline-based compounds that showed a beneficial effect in models of acute or chronic inflammatory conditions were given by sc injection (Finkin-Groner et al. 2015, 2017; Shifrin et al. 2016, 2017). This route was also used for chronic administration of AN1284 via subcutaneously implanted mini-pumps in diabetic mice with kidney and liver damage, to avoid the stress of daily injection (Permyakova et al. 2020). We now show that after its elevation by LPS, a sc injection of 0.5 mg/kg produced a maximal reduction of 31–38% in IL-6 in the plasma, brain, and liver and in TNF-α in the plasma. The same dose also reduced IL-6 in the liver by 35% when given by gavage. However, to decrease this cytokine in brain and plasma by similar amounts, it was necessary to give 2.5 mg/kg.

AN1284 is rapidly metabolized by preparations of mouse and human liver microsomes. Thus, using LC–MS/MS analysis, we identified the indole, AN1422, as a major metabolite in blood and tissues of mice. This confirms a similar indoline to indole oxidative metabolism reported by Sun et al. (2007), who showed that several different cytochrome P450 enzymes, including CYP2C19 and CYP3A4, can convert indolines to indoles. AN1422 has an anti-inflammatory activity in cells, but at none of the concentrations tested did it reach the efficacy of AN1284. This accords with our previous finding that indole derivatives are less potent as anti-inflammatory agents than indolines (Yanovsky et al. 2012; Furman et al. 2014).

As might be expected with a lipophilic compound (Lin and Lu 1997), AN1284 was rapidly absorbed after sc administration and reached 6- and 21-fold higher concentrations, respectively in the brain and kidneys than in the plasma and liver. This explains why a dose of 0.05 mg/kg caused a significant reduction of IL-6 of 22.3% in the brain but not in the plasma or liver. The ratios of the peak concentrations of the indole metabolite to those of the parent drug were similar in plasma and tissues.

By contrast, oral administration of AN1284 (2.5 mg/kg) resulted in 47-fold higher Cmax in the liver than in plasma. This was only 2- and sixfold higher in the brain and kidneys. This accords with the finding of a maximal reduction of IL-6 in the liver at 0.5 mg/kg, the lowest dose tested. Comparison of the AUCt of AN1284 in the plasma by the two routes showed that oral administration reduced the bioavailability to 2.4% of that given by injection. Plasma and tissue levels of AN1284 declined rapidly when given by either route, with a half-life in plasma of only 20 min. After oral administration, the amounts of the indole metabolite in plasma and kidneys, relative to those of the parent drug, were almost double those after seen after sc injection, but they were not different in the liver. In the brain, Cmax and AUC were very similar for AN1284 and AN1422. The amount of AN1422 in the brain represented 11.2% of the peak level found in the liver, while that of AN1284 was only 4.2%. On the other hand, after sc injection, the % in the brain of the peak hepatic concentration was similar for the parent drug and metabolite. Assuming that most of the metabolic conversion of AN1284 to AN1422 takes place in the GIT and liver, the relatively high brain levels of the metabolite may be explained by the fact that it is even more lipophilic than AN1284. This would speed its entry into the brain and slow its egress, as indicated by the increase in the half-life of both compounds after oral and sc administration of AN1284.

We only gave one dose of AN1284 by injection and a five-fold higher dose by mouth. Thus, we do not know whether the increase from 0.5 mg to 2.5 mg/kg would give the same incremental increases in blood and tissue levels by each route. However, the finding that the plasma concentration of AN1284 (2.5 mg/kg) was actually lower when given by gavage than after 0.5 mg/kg by injection suggests that much of the drug was removed before reaching the circulation. This can be explained by the passage of the entire blood supply of the upper gastrointestinal tract (GIT) through the liver before reaching the systemic circulation (Leahy et al. 2000). The GIT and liver contain large amounts of the enzyme, CYP3A, which is known to play a role in the first-pass metabolism of many orally dosed drugs (Kato 2008).

Indapamide is another indoline that is oxidized by liver microsomal enzymes to the indole and a 5-OH derivative (Sun et al. 2009). In the rat, indapamide is converted to the 2- and 3- and 5-OH metabolites, together with their glucuronides (Klunk et al. 1983). In the current study in mice, we found that AN1284 is oxidized to a 7-OH metabolite and its glucuronide (Fig. 4). If other metabolites were formed, they were below the limits of detection. No evidence was found for oxidation in any other part of the molecule or of the indole, AN1422.

Glucuronidation is an enzyme reaction process catalyzed by UDP-glucuronosyl transferases and is often a secondary step after metabolites are produced by phase I reactions such as hydrolysis, hydroxylation, and dealkylation (Yang et al. 2017). As shown for many other compounds, glucuronides are formed in the GIT and liver and stored in the gall bladder from which they can be released from the bile to the gut, excreted in the feces or undergo recycling to the parent drug (Roberts et al. 2002). Relatively high levels of the 7-OH metabolite and its glucuronide were already formed in the liver, 5 min after oral administration of AN1284, and explain the presence of larger amounts of this metabolite in the urine. After sc injection of AN1284 (0.5 mg/kg), the 7-OH derivative and its glucuronide were below the level of...
detection in the liver but were measurable in the 24-h urine samples.

We had previously shown that chronic administration of AN1284 to diabetic mice at a dose of 2.5 mg/kg/day by subcutaneously implanted mini-pumps resulted in a concentration in the kidneys of 140 ng/g (Permyakova et al. 2020). This was sufficient to reduce significantly the elevated protein expression of inflammatory markers MCP1, TNF-α, TGF-β, and IL-18 and prevent the severe enlargement of the glomerulus and of Bowman’s capsule. As expected, the drug levels in the liver produced by this method of administration were much lower (36.4 ng/g) and represented only 9.3% and 12.7% respectively, of the peak hepatic and renal levels seen after injection of a single dose of 0.5 mg/kg. Nevertheless, they were still sufficient to decrease hepatocellular damage, liver triglyceride, and cholesterol contents (Permyakova et al. 2020). In preliminary experiments in mice with existing fatty liver disease, we found that AN1284 (1 mg/kg/day) given by this route was sufficient to reduce steatosis and fibrosis. When administered in the drinking fluid at concentrations of 0.5 and 1 mg/kg/day in the current study, hepatic levels of AN1284 were 26 and 36 ng/gm, similar to those achieved by 1 and 2.5 mg/kg/day, respectively, by subcutaneously implanted mini-pumps. These are only 1.6–2.4% of the peak levels seen after a sc injection of AN1284, 0.5 mg/kg, a fivefold higher dose of AN1422 still did not have a significant effect. Thus, it is unlikely that the indoline metabolite contributes to or detracts from any potential therapeutic effect of AN1284 in fatty liver disease. We have no information about the activity of AN1280, the 7-OH metabolite in vivo. It is converted so rapidly to a glucuronide that it is doubtful whether it could affect the activity of AN1284.

We do not know whether AN1284 undergoes similar metabolic changes in other species or in human subjects. Even if it does, it should not be necessary to increase the dose to obtain therapeutic concentrations for treating chronic liver disease, unlike other drugs that undergo first-pass metabolism in the liver. It remains to be determined whether oral administration of AN1284 can also reverse liver damage in human subjects with this condition.
Conclusions

AN1284 is a novel, indoline derivative with anti-inflammatory activity at picomolar concentrations. It can prevent tissue damage in a mouse model of chronic liver and kidney disease when given by sc implanted mini-pumps. AN1284 also shows anti-inflammatory activity in the liver after oral administration at a dose similar to that after sc injection but at a five times higher dose is required to reduce IL-6 in the brain and plasma. The several-fold higher concentrations of unchanged drug found in the liver than in plasma and other organs make it an ideal drug for treating liver disease.

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Author contribution All authors read and approved the manuscript and all data were generated in-house.

MaW: Conceptualization; supervision, calculations, writing.
CB, MM, TW: all biological experimentation.
IY, SZ: chemical syntheses.
MiW: Analytical and mass spectral studies.
AN: Supervision of spectral analyses and chemical syntheses.

Availability of data and material All data are available in supplementary files S1-S8.

Declarations

Ethics approval All experiments were performed according to the ethical committee guidelines of The Hebrew University Medical School, numbers MD-18–15512-4 and MD-20–16205-2.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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