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COMPARISON OF TWO CLASSES OF NON-PEPTIDE DRUGS AS ANTAGONISTS OF NEUTROPHIL RECEPTORS FOR f-Met-Leu-Phe

PYRAZOLONS AND IODINATED RADIOGRAPHIC CONTRAST AGENTS

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Abstract—The radiographic contrast agent sodium diatrizoate (DTR) reportedly inhibits f-Met-Leu-Phe-induced chemotaxis in human neutrophils. DTR is also an ingredient of Ficoll-Paque, a density centrifugation medium widely used to purify human polymorphonuclear leukocytes (PMNs). Exposure of PMNs to DTR during preparation had no detrimental effect on subsequent binding characteristics of tritiated f-Met-Leu-Phe, probably owing to a rapid dissociation of DTR from the PMN receptors. DTR competed directly with f-Met-Leu-Phe for receptor binding, but was 160-and 640-fold less potent than phenylbutazone and 1,2-diphenyl-4-[3-(1-naphthyl)-propyl]-3,5-pyrazolidinedione (DPN; an analog of phenylbutazone), respectively. Iohexol and the methylamide of DTR did not compete with [^{3}H]f-Met-Leu-Phe in receptor binding, supporting the existence of a definite interaction between iodinated aromatic molecules and the f-Met-Leu-Phe receptor. DTR did not inhibit prostaglandin synthesis, as did DPN. Both drugs inhibited chemotactic peptide-induced release of superoxide anion in a concentration-dependent manner, and were relatively selective for f-Met-Leu-Phe, as opposed to C5a. Both drugs at 10 μM interfered non-selectively with chemotactic peptide-induced β-glucuronidase release from PMNs. Available non-peptide antagonists of f-Met-Leu-Phe exhibited other pharmacodynamic properties that could make them unsuitable for future *in vivo* studies designed to probe the physiological role of the receptor.

A vast body of knowledge has accumulated on the effects and mechanism of action of formylated tripeptide f-Met-Leu-Phe on phagocytic leukocytes [1]. The receptor for f-Met-Leu-Phe from HL-60 cells has been cloned recently [2, 3] and shown to belong to the rhodopsin superfamily. This finding is consistent with the central role of G proteins in its signal transduction mechanism and with the modulatory effect of GTP on the binding characteristics of f-Met-Leu-Phe [4–6].

These advances in receptor characterization contrast with the paucity of data concerning the physiological ligand(s) and the role of these receptors. The agonist f-Met-Leu-Phe, as well as the peptide antagonists [prototype Boc-Phe-D-Leu-Phe-D-Leu-Phe (Boc-FlFlF†)], are the result of purely empirical structure–activity programs based on an assumption that bacterial and mitochondrial proteins, that possess a formylated Met residue at the NH_2 terminus, would be recognized directly by phagocytic cells [1, 7, 8]. Whether an endogenous hormone exists that binds to this receptor is an alternate, but unproven possibility; the physiologic role of receptors specific for f-Met-Leu-Phe remains largely unexplored.

That several unrelated drugs interact with receptors for f-Met-Leu-Phe is of interest to pharmacologists. The receptor is occupied and activated by several non-formylated peptides including bacitracin and other peptide antibiotics [9]. By contrast, the pyrazolon drugs phenylbutazone (PBZ) and sulfinpyrazone behave as competitive antagonists in binding and functional tests involving neutrophils [10–12]. PBZ does not inhibit the binding of radiolabeled C5a, a chemotactic peptide possessing distinct receptors [11]. Structure–activity studies have shown that this property is distinct from the capacity of these drugs to inhibit cyclooxygenase, but is well correlated with the partition coefficient in octanol [13]. The most potent antagonist identified in the pyrazolon class was 1,2-diphenyl-4-[3-(1-naphthyl)-propyl]-3,5-pyrazolidinedione (DPN) [13]. Other reports suggest that various non-steroidal anti-inflammatory drugs (NSAIDs) can antagonize f-Met-Leu-Phe at high concentrations: diclofenac [14], indomethacin [15], meclofenamic acid and ibuprofen [16], to name a few. Much of this literature was not critical on the concentration used relative to the *in vivo* therapeutic levels, and it is clear that many other NSAIDs do not interact with f-Met-Leu-Phe receptors [16].

Rasmussen *et al.* [17] have proposed that a radiographic contrast medium, sodium diatrizoate

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† Abbreviations: Boc-FlFlF, Boc-Phe-D-Leu-Phe-D-Leu-Phe; DMA, diatrizoic methyl amide; DPN, 1,2-diphenyl-4-[3-(1-naphthyl)-propyl]-3,5-pyrazolidinedione; DTR, sodium diatrizoate; HBSS, Hanks' balanced salt solution; NSAID, non-steroidal anti-inflammatory drug; PBZ, phenylbutazone; PGE_2, prostaglandin E_2; and PMN, polymorphonuclear leukocyte.

(DTR; Urografin, Hypaque), may be a competitive antagonist of f-Met-Leu-Phe, based solely on *in vitro* inhibition of the chemotaxis. The effect was clearly related to a defined chemical structure as related iodinated contrast agents, such as iohexol, failed to inhibit f-Met-Leu-Phe-induced chemotaxis. Oddly, DTR is also a key component of the density centrifugation medium Ficoll-Paque routinely used for neutrophil separation by many investigators who have studied f-Met-Leu-Phe binding and functions.

The present study was designed to compare the antagonist properties of the available non-peptide drugs for f-Met-Leu-Phe using a binding assay and some *in vitro* functional assays. It is worth addressing the possibility of a common toxicologic domain for unrelated drugs that would modulate f-Met-Leu-Phe receptor functions, as this property may be avoided in future drug design. On the other hand, the selection or development of non-peptide ligands for f-Met-Leu-Phe receptors is also an interesting goal, as such compounds would be valuable tools to probe the role of this pharmacologic entity *in vivo*. A secondary objective was methodological: the possible detrimental effect of DTR present in the density centrifugation medium on subsequent binding studies involving labeled f-Met-Leu-Phe has been evaluated.

MATERIALS AND METHODS

Drugs and reagents. The following reagents were purchased from the Sigma Chemical Co. (St. Louis, MO): f-Met-Leu-Phe, Boc-FlFlF, Met-Leu-Phe, 2-mercaptoethanol, sodium diatrizoate [DTR; 3,5-bis(acetylamino)-2,4,6-triiodobenzoic acid sodium salt], PBZ, cytochrome *c* (horse heart type VI), cytochalasin B, superoxide dismutase (from bovine erythrocytes), human recombinant C5a and phorbol myristate acetate. Other reagents used were Ficoll-Paque, Ficoll-400 and Dextran T-500 (Pharmacia, Baie d'Urfé, Canada), Hanks' balanced salt solution (HBSS) without calcium, magnesium and phenol red (GIBCO Laboratories, Grand Island, NY), Omnipaque-350 (a solution of iohexol, 755 mg/mL, and other ingredients; Winthrop Laboratories, Aurora, Canada) and DPN (Alfred Bader Chemical Library of Rare Chemicals, Milwaukee, WI).

For binding competition assays and functional assays, drugs were generally dissolved in saline or a physiological buffer. f-Met-Leu-Phe and PBZ were initially dissolved in a small volume of Na_2CO_3 and DPN, Boc-FlFlF, cytochalasin B and the methyl amide of diatrizoic acid (see below) were initially dissolved in dimethyl sulfoxide (DMSO) and further diluted with the appropriate buffer.

Synthesis of diatrizoic methyl amide (DMA). DMA [*N* - methyl - 3,5 - bis(acetylamino) - 2,4,6 - triiodobenzamide] was prepared in our laboratory to test whether altering the carboxylic acid function would change the apparent antagonist behavior of DTR against f-Met-Leu-Phe. Four synthetic steps were involved (Fig. 1); the purity of each compound was tested by thin-layer chromatography, and its molecular structure confirmed by infrared spectroscopy. ^{1}H-Nuclear magnetic resonance (200 MHz, solvent $CDCl_3$:DMSO mixture) and mass spectrometry for DMA. *N*-Methyl-3,5-dinitrobenzamide (compound **1** of Fig. 1) was prepared in 78% yield according to the procedure reported by Percec [18]. Next 5.5 g of **1** (25 mmol) was dissolved in dry methanol and 500 mg of Pd/C (10%) was added. The mixture was agitated under a hydrogen atmosphere (Parr bomb) for 2.5 hr. The mixture was filtered on silica gel and the filtrate evaporated under vacuum. The resulting *N*-methyl-3,5-diaminobenzamide (compound **2**, Fig. 1) was pure enough (>95%) to proceed to the next step. *N*-Methyl-3,5-diamino-2,4,6-triiodobenzamide (compound **3**, Fig. 1) was prepared in 51% yield according to Wallingford *et al.* [19] by treating compound **2** dissolved in 1 M HCl with a solution of 2 N potassium iododichloride; 2.7 g of **3** (4.9 nmol) was diacetylated with an acetic anhydride–sulfuric acid mixture [20]. The reaction yielded 2.4 g DMA.

Human neutrophil isolation. Neutrophils were prepared from heparinized venous blood from healthy volunteers according to Böyum [21] with some modifications. After a 30-min Dextran T-500 (6% solution) sedimentation of erythrocytes, the leukocyte-rich plasma was layered over Ficoll-Paque and centrifuged for 15 min at 350 *g* and 25°. To monitor the eventual detrimental effect of the sodium diatrizoate content of Ficoll-Paque on [^{3}H]f-Met-Leu-Phe binding, an alternate formulation of density centrifugation medium was developed. It was based on iohexol (Omnipaque), a contrast medium reported not to affect f-Met-Leu-Phe functions [17]. The "Ficoll-iohexol" solution was composed of 18.4% (v/v) Omnipaque-350 in 0.9% saline with 4.75% (w/v) Ficoll-400. The polymorphonuclear pellet was purified from contaminating erythrocytes by hypotonic lysis (30 sec) and isotonicity was restored with hypertonic HBSS (10×). PMNs were washed three times (unless indicated otherwise) and resuspended in HBSS, pH 7.4, at a concentration of 1×10^7 cells/mL for the binding assay, at 1.25×10^7 cells/mL for superoxide production analysis, and at 2×10^7 cells/mL for β-glucuronidase assay. PMNs prepared with Ficoll-Paque contained more than 95% neutrophils and were >95% viable by Trypan Blue exclusion, while the preparation from Ficoll-iohexol contained approximately 90% neutrophils and the viability was over 95%.

Radioligand binding assay on intact PMNs. Formyl-L-methionyl-L-leucyl-L-phenylalanine, *N*-[phenylalanine-ring-2,6-^{3}H(N)]-, thereafter designated [^{3}H]f-Met-Leu-Phe (DuPont Canada, Markham Canada; sp. act. 53.6 or 56.4 Ci/mmol), was diluted to a final concentration of 5×10^{-7} M in HBSS containing 0.1% 2-mercaptoethanol and aliquoted in siliconized glass tubes. Aliquots were stored at −80° until used.

The binding assay on intact PMNs was carried out according to Tennenberg *et al.* [22]. In a melting ice bath, binding was executed in triplicate 12 × 75 mm polypropylene test tubes containing the following: HBSS, pH 7.4; [^{3}H]f-Met-Leu-Phe (2.5 to 50 nM); and PMNs (2×10^6 cells). Non-specific binding was determined in a parallel set of matching tubes with an excess of unlabeled f-Met-Leu-Phe (10^{-4} M final concentration). Scatchard plots were constructed using data derived from matching lots of PMNs

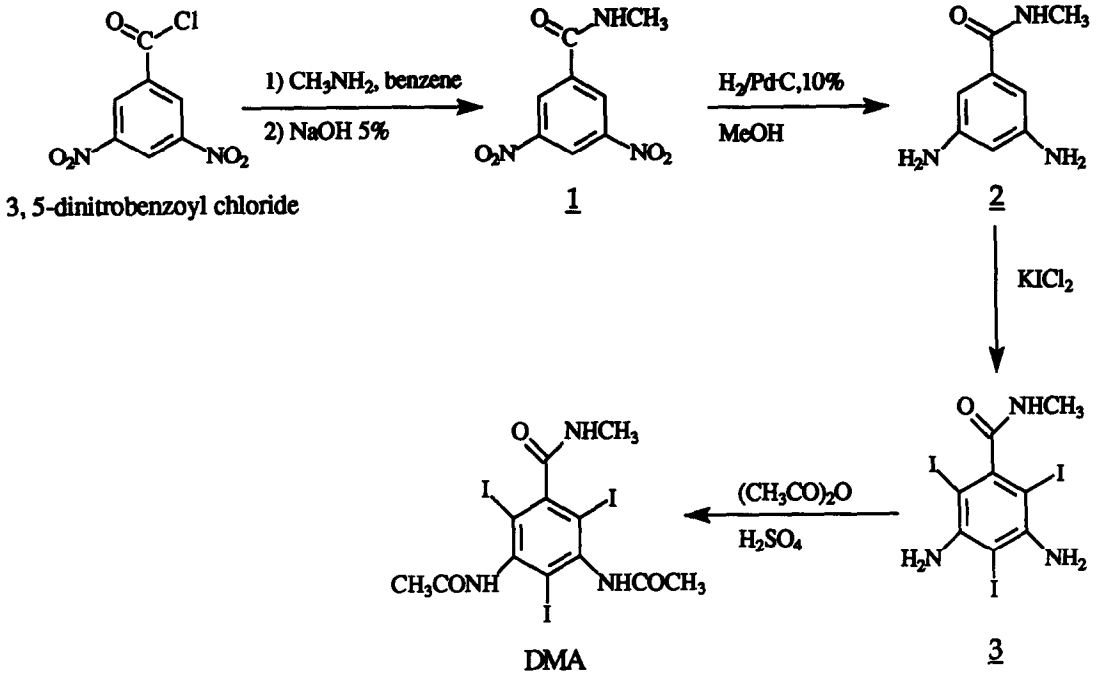

Fig. 1. Schematic representation of the synthesis of DMA.

isolated with Ficoll-Paque or Ficoll-iohexol and submitted to 0–3 washings after the hypotonic lysis of red blood cells. Another series of experiments assessed the capacity of several drugs to compete for [^{3}H]f-Met-Leu-Phe (5 nM) binding to PMNs that were isolated with Ficoll-Paque and washed three times subsequently.

The total volume for binding assays was 1.0 mL. After a 45-min incubation, PMN-bound radioligand was separated from the unbound fluid phase by simultaneous, rapid vacuum filtration through glass fiber filters (Whatmann GF/C, Brandell Corp., Gaithersburg, MD) utilizing a 24-channel cell harvester (model M-24, Brandell Corp.). Filters were washed four times with three 4-mL vol. of ice-cold isotonic saline (pH adjusted to 7.4 with NaOH), removed from the harvester template, and placed into scintillation vials. Eight milliliters of Ecolite + scintillation fluid (ICN Biomedicals, Irvine, CA) was added to vials, and radioactivity was quantified in a Beckman LS 8000 scintillation counter. Separate measurements of total added [^{3}H]f-Met-Leu-Phe were determined by counting in triplicate scintillation vials with the appropriate volumes of the ligand stock solution used in the assay.

Superoxide anion and β-glucuronidase release by f-Met-Leu-Phe-stimulated PMNs. Superoxide production was monitored as the reduction of cytochrome *c* for 5 min at 37° essentially as described previously [13]. β-Glucuronidase release was measured in a 0.5-mL volume of 0.9% saline containing 5×10^6 PMNs and cytochalasin B (5 μg/mL). When a f-Met-Leu-Phe antagonist was used, it was also present at the beginning of the incubation. The reaction mixture was incubated for 5 min at 37° before adding f-Met-Leu-Phe (10^{-7} M final concentration) under a small volume. The tubes were further incubated at 37° for 30 min and then centrifuged at 12,000 *g* for 5 min. Four hundred microliters of the supernatant was transferred in another set of tubes for the β-glucuronidase assay. To this source of enzyme, 200 μL of phenolphthalein glucuronidate (0.01 M) and 400 μL of sodium acetate buffer (0.2 M), pH 4.9, were added. The reaction mixtures were incubated at 56° for 2 hr and the reaction was stopped by adding 3.0 mL of 2-amino-2-methyl-1-propanol buffer (0.1 M), pH 11, containing 0.2% sodium lauryl sulfate (all reagents from the 325-A Kit, Sigma). The absorbance of phenolphthalein was read at 550 nm in the resulting solution.

For either superoxide or β-glucuronidase release assay, the conditions tested included the direct drug-induced superoxide release (possible agonist action) and the inhibitory effect of drugs on f-Met-Leu-Phe-induced release (antagonist action). The selectivity of the antagonism exerted by drugs was tested by using C5a as an alternate stimulus for superoxide production.

Prostaglandin (PG) release inhibition by candidate f-Met-Leu-Phe antagonists. Pyrazolon antagonists of f-Met-Leu-Phe share in common with PBZ the capacity to inhibit PG synthesis [13]. To detect possible inhibitory effects of radiographic contrast agents on this biochemical pathway, the influence of DTR and other drugs on prostaglandin E_2 (PGE_2) release from cultured rabbit dermal fibroblasts was monitored as described previously [13].

RESULTS

Binding of [^{3}H]f-Met-Leu-Phe to intact PMNs as

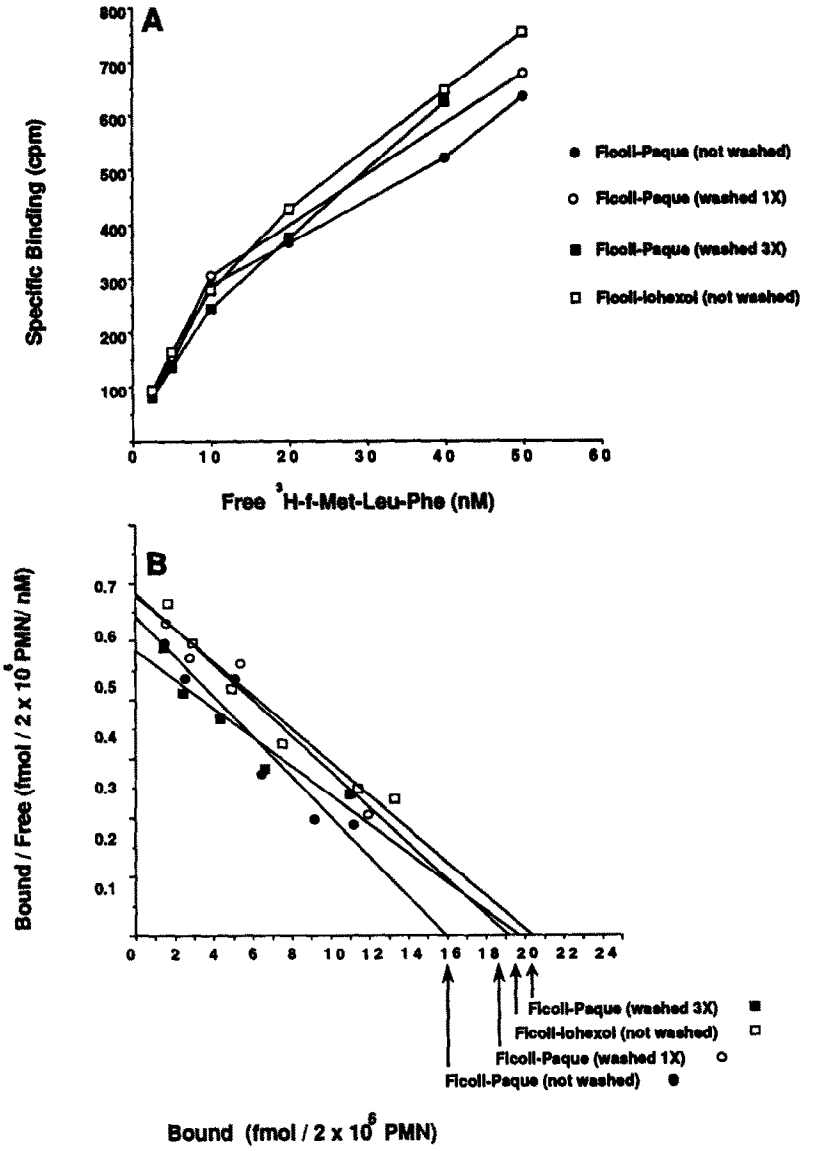

Fig. 2. Binding of [^{3}H]f-Met-Leu-Phe to intact human PMNs as a function of the cell preparation procedure. The experiments were performed simultaneously with the cells of a single donor. (A) Specific binding in the 2.5 to 50 nM range of tritiated ligand. (B) Scatchard plot analysis derived from the same data and assuming a single class of binding sites. Correlation coefficients ranged between −0.94 and −0.98. K_D and B_{max} values derived from the Scatchard analysis are reported in the text. Points represent the specific binding determined in triplicate. The preparation conditions were: Ficoll-Paque separation followed by hypotonic lysis of red blood cells and 0, 1 or 3 washings in HBSS, or "Ficoll-iohexol" separation without washing after hypotonic lysis.

a function of isolation conditions. [^{3}H]f-Met-Leu-Phe receptor binding was established by exposing intact human PMNs to 2.5–50 nM tritiated peptide (Fig. 2). The influence of DTR, a putative antagonist of the formylated peptide [17] present in the formulation of Ficoll-Paque, was evaluated by either washing the cells one or three times after hypotonic lysis or by separating the cells using an alternate Ficoll-iohexol medium (see Materials and Methods). Iohexol is an iodinated compound related to DTR but it does not inhibit chemotactic responses to f-Met-Leu-Phe [17]. The binding characteristics obtained for cells prepared with Ficoll-Paque with 0, 1 or 3 washings after hypotonic lysis were similar, exhibiting only small increases of K_D and B_{max} as a function of washing number (K_D values of 25, 28 and 39 nM and extrapolated B_{max} values of 16, 19 and 22 fmol/2 × 10^6 cells; Scatchard plots, Fig. 2B). Similar values were also obtained with cells prepared using Ficoll-iohexol and not washed following hypotonic lysis (K_D 30 nM, B_{max} 20 fmol/2 × 10^6 cells). The following experiments were carried out

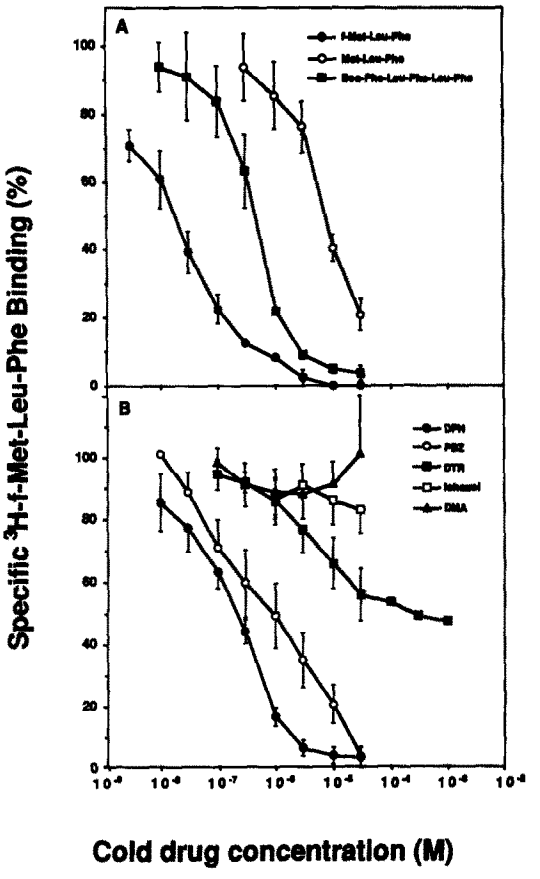

Fig. 3. Competition for [^{3}H]f-Met-Leu-Phe (5 nM) receptor binding to human intact PMNs by (A) peptides and (B) non-peptide drugs. Values are the means, and vertical bars are the SEM, of 1–7 triplicate determinations in cells from different donors. The ordinate scale is the residual binding (%) relative to the total binding without competing drug established for each donor. The non-specific binding was subtracted.

using Ficoll-Paque separation and the cells were washed three times subsequently.

Competition for [^{3}H]f-Met-Leu-Phe receptor binding on intact PMN by various drugs. The tritiated ligand at 5 nM was effectively displaced when the cold f-Met-Leu-Phe concentration was raised (IC_{50} 1.9×10^{-8} M) (Fig. 3A). Non-specific binding was subtracted in competition experiments and was below 10% of the total binding at the concentration of tritiated ligand used. The identity of the binding site with a functional receptor for f-Met-Leu-Phe is consistent with the low potency of a non-formylated peptide, Met-Leu-Phe, and with the capacity of a known antagonist, Boc-FlFlF, to compete for binding (IC_{50} 7.2×10^{-6} and 4.4×10^{-7} M, respectively). PBZ and its analog, DPN, were the most potent non-peptide drugs to displace binding (IC_{50} 8.7×10^{-7} and 2.2×10^{-7}, respectively; Fig. 3B). Iodinated radiographic contrast agents did not compete for the tritiated peptide binding to the same extent as the peptides and NSAIDs tested (Fig. 3B): only DTR inhibited a significant fraction of the binding above 10^{-6} M (IC_{50} 1.4×10^{-4} M). Its amide, DMA, and iohexol were not active in this respect in the concentration range tested.

Functional studies on non-peptide antagonists of f-Met-Leu-Phe. The most active non-peptide competing agents from each drug class, DPN and DTR, were applied to functional tests. The release of superoxide anion in neutrophils stimulated by the chemotactic peptides f-Met-Leu-Phe or C5a was used as an assay for putative f-Met-Leu-Phe antagonists. Both DTR and DPN exerted a concentration-related antagonist effect against f-Met-Leu-Phe (Table 1). DPN was clearly more potent than DTR, exerting significant antagonism at and above 10^{-7} M; DTR exerted only a partial inhibitory effect at 10^{-4} M. The non-peptide drugs were not totally selective, because DPN at 10 μM inhibited significantly the superoxide release induced by C5a. By comparison the peptide antagonist of f-Met-Leu-Phe, Boc-FlFlF (10^{-5} M), exhibited a good selectivity for the formylated peptide. The drugs alone were not significant stimulants for the release of superoxide, supporting the previously reported pure antagonist status of DPN and DTR [13, 17]. The same set of drugs failed to inhibit the release of superoxide from PMNs stimulated by a receptor-independent agent, phorbol myristate acetate (10^{-6} M) (not shown).

The release of preformed granular material is also a consequence of stimulation by chemotactic peptides. β-Glucuronidase is present in lysosomal granules in PMNs [24]. Both drugs DPN and DTR (10^{-5} M) inhibited significantly the release of β-glucuronidase induced by f-Met-Leu-Phe (Table 2). However, both drugs appeared to directly release the enzymes in the absence of chemotactic peptides, and their inhibitory effects were not totally selective as a significant inhibition of C5a-induced enzyme release was also noted. By contrast, Boc-FlFlF had no direct secretory effect on the assay and behaved as a selective antagonist of f-Met-Leu-Phe.

PBZ and its congener, DPN, inhibited the spontaneous PGE_2 release from cultured fibroblast, the former pyrazolon drug being more potent than the latter (Table 3). DTR was not active in this respect (10^{-6} or 10^{-5} M).

DISCUSSION

In a previous study, DTR was identified as one radiographic contrast agent capable of inhibiting f-Met-Leu-Phe-induced chemotaxis of human PMNs [17]. The evidence presented was only of a functional nature. We confirm that DTR is a f-Met-Leu-Phe antagonist, but that it exhibits low potency and selectivity.

The presence of DTR in the density centrifugation medium Ficoll-Paque did not alter subsequent [^{3}H]-f-Met-Leu-Phe binding significantly. We applied a Scatchard analysis on intact PMNs, a procedure inspired by Tennenberg *et al.* [22], who have demonstrated a single class of binding sites with affinity and capacity similar to the ones that we have obtained. Substituting iohexol for DTR in the density centrifugation medium had no significant effect on binding parameters. The number of washings after

Table 1. Effects of inhibitory drugs on the release of superoxide anion from neutrophils stimulated with chemotactic peptides

Drug	Concentration (M)	Ferricytochrome *c* reduced (nmol/tube)		
		Superoxide release stimulus*		
		None	f-Met-Leu-Phe	C5a
None (control)		0	11.85 ± 0.29	9.63 ± 0.12
DTR	10^{-4} M	0.13 ± 0.06	10.32 ± 0.18‡	10.01 ± 0.14
	10^{-5} M	0 ± 0.19	11.43 ± 0.39	10.72 ± 0.07
	10^{-6} M	0.19 ± 0.26	11.52 ± 0.47	10.67 ± 0.05
DPN	10^{-5} M	0.15 ± 0.07	0.75 ± 0.12§	1.76 ± 0.16§
	10^{-6} M	0 ± 0.04	0.38 ± 0.07§	8.63 ± 0.20
	10^{-7} M	0 ± 0.25	7.40 ± 0.45§	10.72 ± 0.07
Boc-FlFlF	10^{-5} M	0 ± 0.26	0.42 ± 0.19§	8.44 ± 0.10
Analysis of variance†		NS	$P < 0.01$	$P < 0.01$

*Results are the means of triplicate determinations ± SEM. The concentration of the stimulants f-Met-Leu-Phe and C5a was 1.5×10^{-7} M. Tubes contained 2.5×10^6 neutrophils. The control absorbance reading (no stimulus, no inhibitory drug) was subtracted from the other readings.

†One-way analysis of variance was applied to the columns of data. When it showed that groups were statistically different from each other, Dunnett's test was then applied to compare the effect of the drug treatments to the control group [23]: ‡$P < 0.05$, and §$P < 0.01$.

Table 2. Effects of inhibitory drugs on the release of β-glucuronidase from neutrophils stimulated with chemotactic peptides

Drug	Phenolphthalein (nmol generated/tube)		
	β-Glucuronidase release stimulus*		
	None	f-Met-Leu-Phe	C5a
None (control)	9.1 ± 2.1	27.3 ± 2.9	40.4 ± 3.4
DTR, 10^{-5} M	15.4 ± 1.9‡	19.8 ± 2.2‡	34.2 ± 4.5§
DPN, 10^{-5} M	15.0 ± 1.5‡	16.6 ± 2.4‡	26.6 ± 1.3‡
Boc-FlFlF, 10^{-5}	9.8 ± 1.6	10.6 ± 1.5‡	40.2 ± 2.9
Analysis of variance†	$P < 0.01$	$P < 0.01$	$P < 0.01$

*Results are the means of triplicate determinations ± SEM. The concentration of the stimulants f-Met-Leu-Phe and C5a was 1×10^{-7} M.

†One-way analysis of variance was applied to the columns of data. Since groups were statistically different from each other, Dunnett's test was applied to compare each drug group to the control group [23]. Significance levels are expressed as follows: ‡$P < 0.01$, and §$P < 0.05$.

Table 3. Effects of drugs on the spontaneous release of PGE_2 by washed cultured fibroblasts

Drug	Concentration (M)	PGE_2 production* (pg/well)
None (control)		400 ± 25
DTR	10^{-6}	372 ± 28
	10^{-5}	363 ± 28
DPN	10^{-6}	363 ± 5
	10^{-5}	189 ± 7‡
PBZ	10^{-6}	251 ± 13‡
	10^{-5}	142 ± 5‡
Analysis of variance†		$P < 0.01$

*Values are the means ± SEM of triplicate determinations.

†One-way analysis of variance was applied to the experimental groups. The groups were found to be statistically different from each other. Dunnett's test was then applied to compare the effect of the drug treatments to the control group [23]: ‡$P < 0.01$.

the hypotonic lysis of red blood cells influenced the assay slightly, although not in the predicted direction. It was predicted that DTR would reduce the apparent affinity of the receptors for f-Met-Leu-Phe and that repeated washings would restore it, if this compound is a competitive f-Met-Leu-Phe antagonist. Although PMNs were exposed to very high DTR concentrations in Ficoll-Paque (9%, w/v), the affinity of DTR for the receptors appears to be so low that DTR probably dissociates completely from the receptors when hypotonic lysis of red blood cells is performed.

We have established competition curves for tritiated f-Met-Leu-Phe binding on human PMNs using a low concentration (5 nM) of the labeled peptide. Under these conditions, both peptide and pyrazolon ligands competed for receptor binding with relative potencies similar to those reported in our previous study [13], but at lower absolute concentrations. The previous study was based on the binding of a high concentration of a fluorescent derivative of f-Met-Leu-Phe quantified by flow-cytometry. Under the present conditions, DPN and PBZ were 640- and 160-fold more active than DTR, respectively, as competitors for f-Met-Leu-Phe receptor binding. The low slope of the competition curve for DTR may also suggest that the competition is of a non-competitive nature.

A definite structure–activity relationship seems to exist for the interaction of iodinated contrast media with receptors for f-Met-Leu-Phe; this was suggested by the previous chemotaxis study [17] and is further supported by the lack of activity of two DTR analogs, iohexol and the methyl amide of DTR (DMA). It is possible that the acidic function of DTR, not present in iohexol or DMA, contributes to f-Met-Leu-Phe receptor binding, as well as its aromatic and lipophilic structure. These features are shared by NSAIDs that bind to this type of receptors, e.g. pyrazolons, indomethacin and diclofenac [13–15].

Both DPN and DTR antagonized f-Met-Leu-Phe-induced release of superoxide anion with a potency consistent with binding competition data. However, neither DPN nor DTR inhibited phorbol myristate acetate-induced release of superoxide, suggesting that the drugs were not radical scavengers and that they did not inhibit biochemical pathways necessary for promoting release. DPN was a significant inhibitor of C5a-induced superoxide release only at 10^{-5} M, a concentration well above the effective level for f-Met-Leu-Phe receptor inhibition. Both non-peptide drugs at 10 μM possessed a small direct stimulatory action on β-glucuronidase release, and inhibited in a non-selective manner the enzyme release induced by either f-Met-Leu-Phe or C5a. The stimulation of enzyme release by drugs is not likely to derive from a receptor agonist effect under these conditions. These non-selective actions of DTR occurred at a concentration that occupies only a fraction of f-Met-Leu-Phe binding (Fig. 2). By contrast, the peptide antagonist of f-Met-Leu-Phe, Boc-FlFlF, appeared to be selective for the tripeptide and not directly active in functional assays involving PMNs. The highly lipophilic drugs DTR and DPN may affect a number of biochemical processes at high concentrations with a number of consequences. DTR is a known inhibitor of platelet aggregation *in vitro* and *in vivo* [25, 26], an example of such possible multiple activities. DTR actions at very high concentrations may be of toxicological interest because such high levels are obtained locally during angiographic and urographic procedures.

Available non-peptide antagonists of f-Met-Leu-Phe exhibit other pharmacodynamic properties that could make them unsuitable for future *in vivo* studies designed to probe the physiological role of the receptor. DPN, the most potent non-peptide antagonist identified, retains a fraction of the cyclooxygenase blocking properties of its congener, PBZ (Table 3), whereas the radiographic contrast agent DTR is not active in this respect. However, DTR is neither sufficiently potent nor selective to be considered as a useful pharmacologic tool. DPN may be used in experiments dealing with f-Met-Leu-Phe antagonism in experimental animals after careful titration of the active dose and with comparative experiments using NSAIDs that are not f-Met-Leu-Phe receptor ligands, such as tolmetin [16].

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