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Determining Selectivity of Drugs by Quantitative Two-Dimensional Gel Analysis

A STUDY OF TENIDAP, PIROXICAM, AND DEXAMETHASONE

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ABSTRACT. *In vitro* pharmacologic measures of drug specificity are well established, i.e. drug interaction with a specific target such as an enzyme, receptor, or ion channel. However, *in vitro* measures of drug selectivity, defined as effects on secondary targets, are lacking. Two-dimensional gel electrophoresis (2-D gel) was examined as a measure of drug selectivity by comparing the effects of three drugs, tenidap, piroxicam, and dexamethasone, on the synthesis of intracellular proteins in lipopolysaccharide (LPS)-stimulated murine macrophages. A set of 902 ^{35}S-methionine-labeled proteins were separated consistently, identified by their coordinates of apparent isoelectric point and molecular weight, and quantified. LPS altered the concentrations of 45 proteins. Tenidap, at 10 μM, affected a total of five proteins (suppressed three; stimulated two), whereas piroxicam, at 10 μM, suppressed two proteins. Dexamethasone at 0.01 μM suppressed eight proteins and stimulated one. Thus, none of the drugs reversed the LPS-induced changes. Two of the eight proteins suppressed by dexamethasone were also suppressed by tenidap and were identified as proIL-1α and proIL-1β. Since the subset of affected proteins provided a unique protein "fingerprint" for each drug, the three drugs were mechanistically differentiated by 2-D gel analysis. Compared to LPS (5% affected proteins), all three drugs were selective (≤1% affected) with piroxicam > tenidap > dexamethasone. With identification of affected proteins, this technique can provide a useful *in vitro* assessment of drug selectivity. BIOCHEM PHARMACOL 52;6:917–925, 1996.

KEY WORDS. drug selectivity; two-dimensional gel electrophoresis; tenidap; piroxicam; dexamethasone; lipopolysaccharide

Traditionally, drugs, i.e. chemicals with a useful pharmacological activity, are discovered by measuring their effects in a specific biochemical protocol such as receptor binding, enzyme rates, ion transport, and smooth muscle contraction, or by an analogous mechanistic approach. A high degree of drug "specificity" for the particular mechanism can be achieved. Thus, piroxicam inhibits the cyclooxygenase enzyme [1], thereby decreasing the synthesis of prostaglandins, but does not inhibit the related lipoxygenase enzyme [2]. Also cimetidine antagonizes binding of histamine to the H-2, but not the H-1 receptor [3].

Even when a drug has great specificity for an enzyme or receptor, that precision may not be reflected in the effects of the drug, i.e. the drug may lack "selectivity." In this paper, we define specificity as a measure of the action of a drug on its primary target and selectivity as a measure of a drug's effect on secondary targets. For example, dexamethasone binds with great specificity to the glucocorticoid receptor, but it cannot be classified as a selective drug. Once dexamethasone binds to the receptor, the receptor–drug complex can now bind to the glucocorticoid response elements (GRE) found in the promoter region of the genes of many proteins [4, 5] and induce multiple transcriptional changes affecting general metabolism and modifying numerous inflammatory reactions. Standard *in vitro* drug-testing paradigms evaluate the specificity of drugs in precise mechanistic assays. However, these tests do not address the broader question of drug selectivity.

Questions of selectivity extend to all drug classes. For example, methods are being developed that target drugs to affect specific transcriptional or translational regulatory molecules. It is likely that a highly specific drug interaction with regulatory molecules such as AP1 [6, 7] will lead to effects on families of co-regulated proteins rather than affecting a single change. In addition, many drugs that are in current use do not have a single defined mechanism. In the arthritis area, for example, drugs such as gold [8], *d*-penicillamine [9], hydroxychloroquine [10], and sulfasalazine [11] provide effective therapy, but the molecular mechanisms of their therapeutic activity remain undefined. Evaluation of drug selectivity may provide an approach to defining mechanistic similarities and differences. Further, because assessment of drug selectivity must be based upon a

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broad survey of potential drug effects, valid measures of drug selectivity should be useful to differentiate drugs by class and also define subtle mechanistic differences within a class. Therefore, we believe it is imperative to develop reliable *in vitro* techniques that will facilitate development of a quantitative picture of drug *selectivity*.

Analysis by 2-D gel§ appeared to us to have the potential to provide a general approach for measuring drug selectivity. First, on the basis of differences in size (M_r) and charge (pI), this technology can separate close to 2000 proteins in a single analysis [12, 13]. Second, computer software has been developed that can analyze and reproducibly match a 2-D gel pattern containing large numbers of individual, identifiable proteins. Third, recent advances permit quantitation and resolution of protein spots by two-dimensional Gaussian curve fitting [13]. These developments enable characterization of individual proteins by size, charge, and relative concentration. In essence, a database can be constructed that is composed of the amounts of a large number of individual proteins under defined conditions [14], and the fate of each protein can be followed under different experimental conditions. The practicality of developing a database of changed proteins has been amply demonstrated for study of proteins during cell proliferation [14], differentiation [15], transformation [16], and after treatment with substances such as interferon [17]. A hepatocyte database has also been constructed for examination of toxicological risk upon exposure to drugs and carcinogens [18, 19].

In an earlier study, Anderson *et al.* [20] suggested that use of 2-D gels might also be extended to the analysis of drug mechanisms. It is logical that the effects of an individual drug could be described by the pattern of proteins whose amounts are altered in the presence of the drug. The pattern of the drug-induced changes would constitute the drug's "fingerprint." The detailed fingerprint could be used to compare similarities and differences between drugs and assess selectivity. Because a sample of ca. a thousand proteins can be examined by 2-D gel methodology as opposed to a few predetermined proteins by more traditional pharmacologic methods, an estimate of drug selectivity can be made. To test this hypothesis, LPS was used to induce the synthesis of macrophage proteins typical of those induced during inflammation. The effects of three drugs, tenidap [21, 22], piroxicam [23, 24], and dexamethasone, each of which modify the inflammation of rheumatoid arthritis, were determined by quantitative 2-D gel analysis and compared. Based on their "fingerprint," each of the three drugs was judged to be mechanistically different, and both piroxicam and tenidap were more selective in their mode of action than dexamethasone.

§ *Abbreviations:* 2-D gel, two-dimensional gel electrophoresis; LPS, lipopolysaccharide; IL, interleukin; proIL-1, pro-interleukin-1; CSP, constitutive standard protein; PSN, protein spot number; pI, isoelectric point; and DMEM, Dulbecco's modified Eagle's medium.

MATERIALS AND METHODS

Chemicals

Tenidap and piroxicam were prepared by S. B. Kadin and by J. G. Lombardino, respectively, of the Central Research Division, Pfizer Inc. Dexamethasone was purchased from the Sigma Chemical Co., St. Louis, MO. Recombinant murine 17 kDa IL-1α (residues 115–270) and IL-1β (residues 118–269) were prepared as before [25, 26]. Goat anti-IL-1α and anti-IL-1β antibodies were prepared by immunization with the purified and biologically active murine cytokines. The anti-IL-1α and the anti-IL-1β antibody reacted selectively with the homologous IL-1α or β on western blot and ELISA analyses [27].

Sample Preparation

Resident peritoneal cells from C3H/HeN mice (Charles River) were suspended, at a concentration of 3×10^6 cells/mL, in RPMI medium with 5% fetal bovine serum (GIBCO Laboratories, Grand Island, NY, or Hyclone Laboratories, Logan, UT), penicillin (100 U/mL), streptomycin (100 mg/mL), and glutamine (2 mM). The cells were left in 35 mm plastic cluster plates (Costar, Cambridge, MA) for 2 hr to become adherent. The medium was then removed, and the adherent cells (~1×10^6) were washed two times with 1 mL of serum-free DMEM without methionine (DMEM-Met$^-$, Gibco). The adhered cells were allowed to rest in the incubator in this medium for 1 hr. The medium was decanted and, to the adhered cells, drugs, prepared in 1 mL of serum-free DMEM-Met$^-$, were added together with 20 μg/mL of refined, purified LPS from *Salmonella minnesota* (RIBI, Immunochem Research, Hamilton, MT). At the same time, 100 μCi of [^{35}S]methionine and sufficient unlabeled methionine to bring the concentration to 65 ng/mL were added. The wells were incubated for 6 hr at 37° in 5% CO_2, washed two times with unlabeled 10 mM sodium phosphate buffer (PBS), and the cells were scraped from the wells into cell lysis buffer consisting of 50 mM KCl, 10 mM NaCl, 50 mM KH_2PO_4, pH 7.5, 1 mM EDTA, 0.5% NP-40, and 10 mM diisopropyl fluorophosphate. Cell debris was removed by centrifugation, and the lysates were transferred to clean tubes and stored at −70° until used.

2-D Gel Electrophoresis of Cell Lysates

2-D gel separations were carried out using the method described by Garrels [13]. The isoelectric focusing gels were 0.8 mm × 20 cm tube gels with pH 3–10 ampholines from LKB (Baltimore, MD), and the second dimension incorporated 1 mm thick 24 × 24 cm 12.5% acrylamide slab gels. Gel loads were adjusted to approximately 400,000 dpm. After electrophoresis, the gels were treated for fluorography with DMSO and 2,5-diphenyloxazole (PPO) [28], and vacuum dried onto filter paper. Multiple exposures of each gel were obtained ranging from 0.6 to 17 days, all of which were done at −70° using Kodak X-OMAT AR film.

Gel Analysis

All films were scanned using a Molecular Dynamics laser scanner, and protein spots were quantified using PDQ-Scan software from Protein Databases, Inc. (PDI, Huntington Station, NY). Gel images were edited, matched, and annotated using PDQUEST™ software (PDI). Sun Microsystems 3/260 and 3/60 computers were used to run both software packages. Data from multiple exposures of each gel were merged together using CALSTRIPS™ (PDI) to create computer images with a vastly greater dynamic range than a single film. Protein spots were quantified using the Gaussian fitting algorithms of the PDQ-Scan software. Proteins were matched between gels of each experiment, and the reference images of each experiment were matched to correlate data from multiple experiments. Apparent molecular weights (M_r) and isoelectric point (pI) assignments were made based on internal standards that were identified by co-migration with known proteins of NIH 3T3 cells in mixing experiments.

To correct for the variability observed between experiments, e.g. differences in incorporation efficiency between samples, variations in the quality and composition of the label, and differences inherent in the primary cells used in this study (activation and priming), spot intensities were normalized to the total counts of a group of CSPs. CSPs were selected to be well resolved and easily separated proteins that exhibited consistent intensities (> 75 dpm) regardless of treatment, and which displayed a range of apparent M_r and pI coordinates covering different locations in the gel. Nine protein spots that met the CSP criteria were selected (Table 1). The absolute disintegrations per minute of these nine CSPs were summed, and their sum (~1400 dpm in an average experiment) divided by 1000 was used to normalize the concentration in a specific protein spot between experiments. In this fashion, the relative intensity of each protein spot was expressed by the following equation:

Relative protein spot size = (observed spot dpm/sum CSPs dpm) · 1000.

Spot quantities were averaged across replicate gels, and treatments were compared and analyzed by Student's *t*-test. Significantly affected protein spots were identified as those that exhibited quantitative differences of 2-fold stimulation or 2-fold suppression with a P value of less than 0.05. Once identified, the protein spots were re-examined on the computer images and directly on films to confirm correct matching and visually corroborate the response to treatment. The list of confirmed affected spots of a given treatment was termed its "fingerprint." Fingerprints were then compared to assess uniqueness, and selectivity of each treatment. Although close to 1800 protein spots per gel could be observed and annotated by 2-D gels, for practical considerations such as spot intensities, spot overlapping, and time required to match protein spots, the analysis was restricted to 902 well-resolved and matched protein spots. Protein spots of low intensity and/or appearing erratically in a few gels were not considered in the analysis. All data presented are the results of at least three separate experiments. Each experiment consisted of at least two gels per condition representing a total of six gels per drug.

TABLE 1. Constitutive standard proteins*

CSP No.	M_r (kDa)	pI	Relative intensity†
CSP0215	27.1	4.85	109
CSP0312	29.2	4.82	210
CSP1337	32	4.95	133
CSP3208	24.1	5.69	109
CSP3222	30.3	5.72	69
CSP4102	18.4	5.99	281
CSP5217	24.2	6.06	98
CSP6202	25.8	6.78	172
CSP7040	12.4	6.78	356

* Constitutive standard proteins (CSPs) constitute a series of well-resolved protein spots that exhibited consistant intensities and displayed apparent M_r and pI coordinates well distributed throughout the gels. CSPs were used to normalize the concentration of all protein spots analyzed as described in Materials and Methods.

† The actual total dpm in the CSPs was 1325. Thus in this representative experiment the relative intensity (observed spot dpm/ΣCSP) · 1000 was 0.75× the actual dpm.

Proteins Identified

The M_r and pI coordinates of IL-1 precursors (proIL-1α and proIL-1β) in the gel were identified by immunoblots as described below. The M_r and pI coordinates for the γ-interferon-inducible heat shock proteins, p73 and p71, were obtained by 2-D gel analysis of lysates derived from γ-interferon-treated murine macrophages [29]. The location of calmodulin, tropomyosin-2, -4, and -5, β-tubulin, combined actin-β and γ, and five heat shock proteins (p100, p91.7, p90, p80, and p73) were obtained from an overlay with NIH 3T3 cells as described by Blose [30].

IMMUNOBLOTS FOR IL-1α AND IL-1β PRECURSORS. Lysates were fractionated by the 2-D gel method of O'Farrell *et al.* [12]. Proteins in the second dimension (SDS slab gel) were transferred to nitrocellulose membrane (western blot) according to the method of Gershoni and Palade [31]. Blots were dried and placed on film (Kodak X-omat AR) for 3–5 days at −70°, and then the film was developed. After autoradiography, the blots were immunoprobed with either goat anti-mouse IL-1α or β followed by alkaline phosphatase conjugated swine anti-goat IgG (Boehringer–Mannheim, Indianapolis, IN). Blots were developed with the Phosphatase Substrate System (Kirkegaard & Perry, Gaithersburg, MD) according to the manufacturer. The radioactive precursors of IL-1α and β were identified by placing developed films over immunoprobed blots.

RESULTS

CSP Normalization of Inter-experimental Data

The use of 2-D gels for detailed quantitation of protein patterns has been reported for single cloned cell lines [14], and in these studies a high degree of inter-gel reproducibility was demonstrated. However, for our comparative study

with antiinflammatory drugs, we chose primary murine peritoneal macrophages instead of an established cell line because we expected a more physiological view of the drug effects. Thus, for example, LPS induced only 5 proteins in the murine monocyte RAW cell line (Otterness *et al.*, unpublished observations) versus 46 in primary macrophages used in this study. Moreover, in earlier studies we had evaluated production of IL-1 by primary murine macrophages and found them responsive to drugs [22, 32]. Unlike experiments with cell lines [14], inter-gel variability in spot intensity between experiments with primary cells was too great to allow sensitive detection of differences in protein responses using raw numbers. Therefore, it was necessary to find a method to normalize data obtained from different experiments. A number of approaches were tried including normalization based on the total dpm incorporated in protein, total dpm in all spots, and total dpm in matched spots. However, CSP spot normalization, that is normalization using selected invariant protein spots as described in Materials and Methods, proved to be the most effective. On the average, CSP normalization reduced the coefficient of variation of matching protein spots between experiments by 52% when compared to the coefficient of variation without normalization. By reducing the statistical variation in protein spot intensity between experiments, the normalization increased the sensitivity for detection of drug-induced changes.

2-D Gel Protein Pattern of the Normal Murine Macrophage

The 2-D gel pattern for the control non-drug-treated gels (control) is presented in Fig. 1. This pattern was highly reproducible. Although topological shifts in location of M_r and pI standards occurred in each gel, they were compensated for in the computer analysis. Nine hundred and two protein spots with consistent apparent M_r and pI coordinates and relative concentrations were chosen for detailed analysis on the basis of 12 replicate control gels. Fifteen of those proteins were identified by name and are presented in Table 2. (The pattern of 300 representative protein spots, including the proteins identified by their name and the proteins affected by the drugs used in this study, are shown in Fig. 2.

2-D Gel Protein Pattern of the LPS-Stimulated Murine Macrophage

The intensities of all 902 protein spots analyzed were averaged among duplicate gels within each experiment, and the control and LPS gel averages were compared to identify the protein spots that were affected significantly by LPS (at least 2-fold stimulation or 2-fold suppression). Nine different experiments were used to determine which proteins were members of the LPS-affected set. The LPS fingerprint comprised a set of 45 affected proteins of which 37 were stimulated and 8 were suppressed (Table 3). PSN 407 and 1313 were identified as proIL-1α and β, respectively. PSN

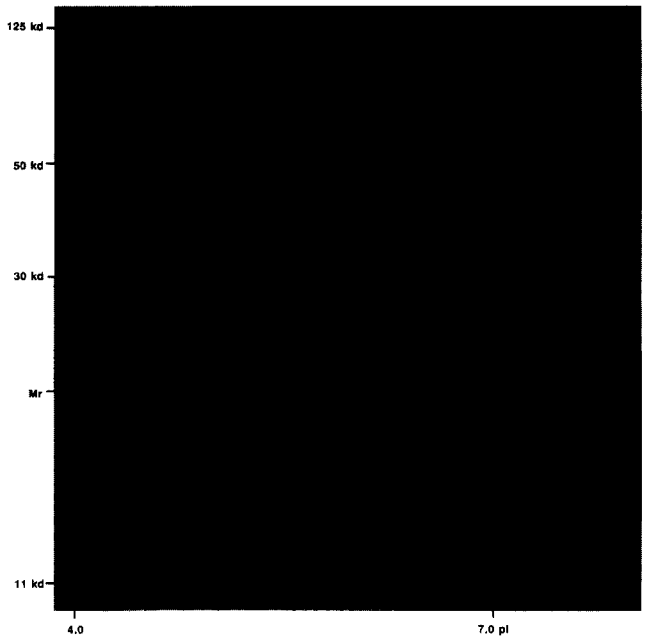

FIG. 1. A typical 2-D gel showing the resolution of spots of ^{35}S-methionine-labeled proteins from LPS-stimulated mouse peritoneal macrophages. The approximate M_r of the proteins is given on the Y axis, and the approximate pI of the proteins is given on the X axis. The pI and M_r values are taken from known proteins running in the gel and not from external standards.

7701 and 7724 were identified as γ-interferon-induced heat shock proteins p73 and p71, respectively (Table 2).

2-D Gel Pattern of Drug Treatment on the LPS-Stimulated Macrophage

The effects of tenidap (10 μM), piroxicam (10 μM), and dexamethasone (0.01 μM) on the LPS-stimulated macro-

TABLE 2. Protein spots that were identified as named proteins*

PSN†	M_r (kDa)	pI	Protein name
140	17.0	4.00	Calmodulin
307	30.0	4.98	Tropomyosin-5
309	29.7	4.86	Tropomyosin-4
407	33.6	4.85	Pro-interleukin-1β
702	100.0	5.01	Heat shock protein; M_r = 1,000,000
1313	29.4	5.23	Pro-interleukin-1α
1337	31.8	5.01	Tropomyosin-2
1703	98.0	5.26	β-Tubulin
1715	91.2	5.14	Heat shock protein; M_r = 90,000
1724	93.9	5.14	Heat shock protein; M_r = 91,700
2506	42.0	5.42	Actin-β and γ combined
2739	71.8	5.50	Heat shock protein; M_r = 73,000
2812	RRR‡	5.45	Heat shock protein; M_r = 80,000
7701	73.3	7.00	γ-Interferon-inducible p73
7724	71.8	7.00	γ-Interferon-inducible p71

* The proteins spots were identified either by immunoblots or by standard gel overlaps containing proteins with known apparent M_r and pI coordinates as described in Materials and Methods.

† PSN = protein spot number given to an annotated protein spot.

‡ RRR = value out of range of internal standards.

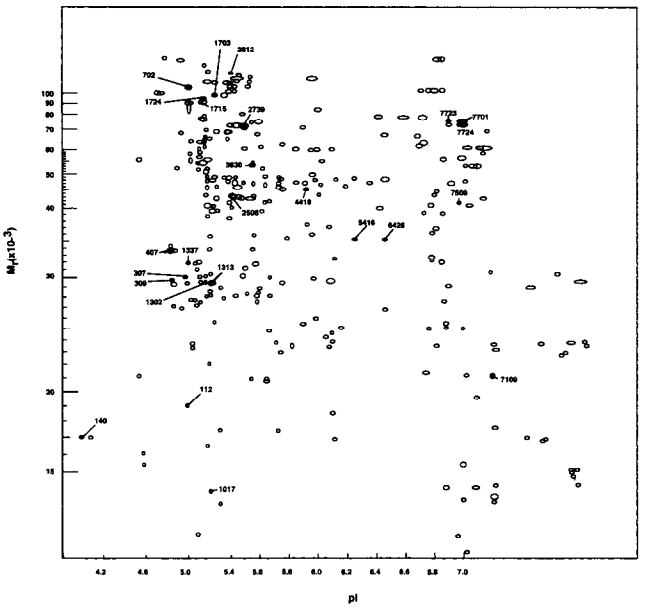

FIG. 2. A computer-generated map of the 2-D gel pattern. The proteins identified by name and all proteins affected by drug treatment are identified by their Protein Spot Number within a background of approximately 300 proteins chosen to be representative of the entire 2-D gel pattern. The spot size and shape roughly reflect that observed in an LPS gel.

phage are also presented in Table 3. The drug fingerprints were defined as the group of proteins having statistically significant differences in concentration in drug-treated and LPS-stimulated cells when compared with the LPS-stimulated cells. Each drug afforded a unique fingerprint. Tenidap treatment of LPS-stimulated macrophages resulted in the suppression of three protein spots (PSN 112, proIL-1β, and proIL-1α) and in the stimulation of two protein spots (PSN 5416 and 6426). The two proteins stimulated by tenidap did not differ between LPS and control gels and, therefore, were not members of the LPS fingerprint set. Piroxicam treatment resulted in the suppression of 2 proteins exclusively (PSN 1017 and 1302); neither protein was a member of the LPS fingerprint. Dexamethasone affected a total of 9 proteins of which 8 were suppressed (proIL-1α, proIL-1β, γ-interferon-inducible p73, PSN 1017, 4419, 7109, 7509 and 7723) and one was stimulated (PSN 3630). Proteins PSN 1017, 3630 and 7109 were not members of the LPS fingerprint set.

Evaluation of ProIL-1α and ProIL-1β as Affected Proteins

Both proIL-1α and proIL-1β were identified by immunoblot as predominant protein spots in gels derived from LPS-stimulated macrophages. The protein spot corresponding to proIL-1α exhibited coordinates of M_r and pI of 29.4 kDa and 5.23, respectively. The M_r and pI values calculated from the amino acid sequence of proIL-1α were 31,024 and 4.88, respectively. Two minor protein spots (PSN 1225 and 1412) were also detected by the immunoblot. The spots appeared as satellites and differed from the main proIL-1α protein species by less than 0.8 kDa in apparent M_r and 0.06 pH units in apparent pI. All three proteins identified as proIL-1α were minor protein spots detected in control gels.

The protein spot corresponding to proIL-1β exhibited coordinates of apparent M_r and pI of 33.6 kDa and 4.85, respectively. The M_r and pI values calculated from the amino acid sequence of proIL-1β were 30,932 and 4.28, respectively. Two minor proIL-1β satellites (PSN 208 and 214) were also detected by western analysis. They differed from proIL-1β in apparent M_r and pI by less than 0.1 kDa and 0.09 pH units. Similarly to the observations with proIL-1α, all three proIL-1β species were minor protein spots in control gels.

After 6 hr of incubation, proIL-1α showed an 11-fold and proIL-1β a 17-fold stimulation by LPS. A 13-fold mean stimulation by LPS was obtained for "total" proIL-1. There was approximately 2-fold more proIL-1α than proIL-1β in the control gels, and this value was not changed significantly by LPS stimulation. Although this ratio (proIL-1α/proIL-1β) was determined exclusively from newly synthesized proteins, a similar ratio was obtained by quantitative immunoblots which measured total mass of both old and newly synthesized IL-1 precursor protein. From the total number of methionines present in proIL-1α and β, and from the assumption that an average protein contains 1.69 methionines per 100 residues [33], the total amount of proIL-1α and β in the extracts was estimated to be 0.03% of total synthesized protein in control experiments and 0.44% in LPS-stimulated experiments.

The stimulation of proIL-1α and β by LPS was suppressed by tenidap and dexamethasone but not by piroxicam (Table 3). This suppression did not appear to cause any significant shift in percentage of proIL-1α or β. Moreover, the satellites responded similarly to the primary proIL-1α or β spot. Using the quantitative radiolabeled method, we found a mean of 54% suppression of intracellular proIL-1 in reasonable concordance with the previous data in which inhibition was determined at 24 hr to be 65% by quantitative western analysis of intracellular IL-1α [34]. With dexamethasone at 0.01 μM, a mean suppression of 44 and 55% were found for proIL-1α and β, respectively.

Discussion

To obtain a highly reproducible and quantitative set of proteins from 2-D gel analysis and examine their changes due to drug treatment, we followed the methods developed by Garrels and coworkers in gel running, quantitation, and data base formation [13, 14, 35]. Close to 1800 spots could be detected on our gels, but approximately half had too few counts to be useful for quantitative analysis. These proteins had either too low a synthetic rate, or too high a rate of secretion or intracellular catabolism to accumulate sufficient counts to be useful for quantitation. The resulting 902

TABLE 3. Set of proteins affected either by LPS or by drug treatment

PSN*	M_r (kDa)	pI	LPS relative intensity†	LPS effect‡	Drug effect on LPS-treated samples§		
					tenidap	Dexamethasone	Piroxicam
112	19.0	4.99	43	1.7	− 2.2		
204	23.6	5.04	184	1.7			
214	33.8	4.80	60	13.4			
proIL-1β	33.6	4.85	699	17.7	− 2.4	− 2.2	
717	55.7	4.55	83	− 1.4			
1017	14.0	5.21	102	0		− 1.6	− 2.5
1122	16.5	5.18	26	− 2.5			
1302	28.5	5.21	128	0			− 1.7
1304	29.4	5.17	170	7.0			
proIL-1α	29.4	5.23	657	10.2	− 2.0	− 1.8	
1412	30.1	5.17	162	5.0			
1505	40.4	5.19	642	2.2			
1622	63.6	5.13	171	6.9			
2101	28.1	5.59	263	2.5			
2425	41.6	5.48	429	2.2			
2723	103.0	5.44	35	4.4			
3216	25.7	5.62	35	− 2.1			
3315	31.6	5.58	169	− 1.6			
3324	28.5	5.72	25	7.6			
3630	53.2	5.56	55	0		1.7	
4112	16.5	5.88	77	3.5			
4407	35.2	5.80	53	− 1.6			
4419	44.0	5.93	55	2.3		− 1.9	
5209	25.0	6.16	51	− 1.3			
5214	24.5	6.10	25	2.1			
5409	47.9	6.26	47	2.7			
5416	35.4	6.17	20	0	2.6		
5440	36.9	6.00	18	− 2.6			
5525	53.9	6.32	23	16.7			
6206	27.4	6.87	64	− 1.9			
6214	29.0	6.68	34	2.8			
6401	41.3	6.75	51	6.3			
6426	35.2	6.49	45	0	3.0		
6506	42.8	6.72	43	2.7			
6520	40.7	6.48	33	20.6			
6620	62.8	6.61	28	5.0			
6703	62.6	6.73	83	2.3			
7021	15.3	>7.0	22	2.9			
7036	15.3	>7.0	637	1.7			
7106	23.4	>7.0	87	1.7			
7109	20.9	>7.0	57	0		− 3.3	
7302	28.9	>7.0	13	6.4			
7406	41.5	>7.0	17	12.4			
7509	40.6	6.98	71	42.9		− 3.8	
7536	52.4	>7.0	32	23.6			
7605	71.9	6.91	58	9.8			
7618	68.0	6.89	15	11.0			
γIFN-p73	73.3	7.00	189	19.9		− 2.1	
7723	73.5	6.90	65	27.2		− 2.6	
γIFN-p71	71.8	7.00	236	12.7			
8527	41.4	>7.0	72	2.5			

* PSN = protein spot number. The PSN numbers for the identified proteins in the table were: proIL-1β, 407; proIL-1α, 1313; γIFN inducible p73, 7701; and γIFN inducible p71, 7724. pI values >7.0 were out of the range of the internal standards.

† LPS relative intensity represents the amount of protein after LPS treatment calculated for each protein by the CSP normalization method described in Materials and Methods.

‡ LPS effect compares the relative amount of protein of control with that of LPS-treated cells. The values quantify the fold change (+ = increase, − = decrease, or 0 = no change) after LPS treatment.

§ Comparison of the effects of the drugs on LPS-treated cells. The values quantify the fold change in relative amount of protein in LPS-exposed cells after drug treatment.

protein spots were sufficient for useful comparative drug "fingerprints" within the practical limits of our 2-D gel analysis.

We limited our drug fingerprint analysis to LPS-stimulated macrophages. We chose these cells as our test system because macrophages have been reported to be cellular targets for these drugs (tenidap and dexamethasone) and because LPS has been shown to stimulate the synthesis

of a number of proteins that are also elevated in inflammatory lesions [36–38]. We used primary cells to achieve a more physiological view of drug effects, and to have a standard against which to evaluate the acceptability of individual cloned cell lines. We used total cellular extracts containing both nuclear and cytoplasmic intracellular proteins hoping to catch those protein changes related to both gene regulation and cytoplasmic responses to stimulation. The analysis was simplified by excluding extracellular proteins that show complex glycosylation ladders and microheterogeneity as secreted polypeptides. Cell stimulation by LPS, or for that matter by most cell stimuli, leads to the formation of early proteins (often acute and transiently expressed, and related to receptor triggering and gene activation) and to very late proteins whose expression is often best observed after 24–48 hr of stimulation. We examined the cells at an intermediate time, 6 hr, hoping to catch an overlapping spectrum of early and late proteins. We did not use secretion inhibitors to enhance intracellular expression of secreted proteins that might otherwise be missed. Therefore, only those newly synthesized ^{35}S-methionine-labeled proteins that reach a significant intracellular concentration 6 hr after LPS stimulation would be included in the analysis.

To set drug concentrations, we required that the drug should have a robust effect against a well-identified pharmacologic endpoint (in this case IL-1 and prostaglandin E) and be at a concentration where the drug shows no evidence of toxicity as measured by lactate dehydrogenase release. From the examination of the fingerprints of the three drugs, it was clear that none of the drugs reversed the effects of LPS stimulation and returned the cell to the control state. Each drug affected a small subset of proteins that did not necessarily belong to the LPS-fingerprint set. Moreover, given that the LPS fingerprint was made up of 45 proteins, only dexamethasone affected a significant percent of those proteins (13%).

We had reason to believe that IL-1α and IL-1β would be key proteins that could be used to distinguish between piroxicam [32] on the one hand, and tenidap [32, 39] and dexamethasone [40, 41] on the other. Specific antibodies against recombinant murine IL-1α and β were used to immunoprobe directly the [^{35}S]methionine nitrocellulose blot transfers after autoradiography. This procedure allowed us to exactly overlay western blots onto radiographs and identify the apparent M_r and pI coordinates for the two ^{35}S-labeled cytokine precursors and their satellites. This method is a general approach for identifying known proteins [42]. The 2-D gel analysis confirmed our expectations; while both dexamethasone and tenidap each suppressed the concentration of proIL-1 in these cells, piroxicam did not. However, dexamethasone and tenidap exhibited different fingerprints, and suppression of IL-1 by dexamethasone was more potent than that of tenidap. In addition, dexamethasone suppressed the amount of γ-interferon-inducible p73, but not p71, although both of the proteins were induced by LPS treatment. Only proIL-1 was common to the fingerprint pattern of the two drugs. These data confirm the obvious conclusion that the two drugs must have different modes of action.

Dexamethasone is likely to affect LPS-induced protein products by both transcriptional and post-transcriptional mechanisms [43]. How tenidap acts in this system is unknown. Under the assay conditions employed, tenidap is expected to induce a change in intracellular pH and to inhibit anion transport processes [44]. The change in pH, in turn, is expected to affect protein synthetic processes as a result of the pH sensitivity of the various protein synthetic components [45]. Likewise, the 6-hr labeling period is sufficiently long that drug-induced changes in protein and/or mRNA degradation rates also could be influenced. Indeed, agents that lower intracellular pH have been shown to selectively increase the rate at which newly synthesized murine proIL-1β is degraded.[1] As a result of this change in ionic homeostasis, therefore, the net effect of tenidap will reflect both a decreased rate of synthetic activity as well as an increased rate of degradative processes. The present results indicate that these changes result in the selective reduction of only a few macrophage proteins.

Both tenidap and piroxicam have, in common, the ability to inhibit the cyclooxygenase pathway of prostaglandin synthesis [1, 46, 47]. Prostaglandin inhibition would be expected to lead to lower cyclic AMP levels in both tenidap- and piroxicam-treated cells and perhaps alter the synthesis of a common set of cyclic AMP-dependent proteins. However, we saw no overlap between the piroxicam and tenidap fingerprints. It is possible that because of the limited experimental window imposed in our study (intracellular proteins prevalent at 6 hr), we could have missed affected proteins that were made very early and degraded, proteins that appeared very late, or proteins that were secreted into the extracellular milieu. Other studies will be needed to determine to what extent the choice of cell, stimulant, and assay time affects the generality of the conclusions presented here.

One further perspective was clear from this study. All three drugs suppressed ca. 1% or less of all proteins studied. Compared with LPS (5% affected proteins), each drug can be viewed as selective. Furthermore, in this study piroxicam affected fewer proteins than tenidap, and both appeared to be more selective than dexamethasone by the 2-D gel criteria. Although the inhibition of IL-1α and IL-1β by tenidap and dexamethasone may provide an explanation for their greater clinical effect when compared with piroxicam in rheumatoid arthritis, the relationship of selectivity to therapeutic and/or side-effect profiles cannot be inferred from this study—it must arise from identification of the functional role of all the affected proteins.

Our ability to distinguish between three drugs of overlapping therapeutic profile suggests that 2-D gel fingerprinting may provide a general approach to evaluate mechanistic differences between drugs. However, very few individual

[1] Gabel C, unpublished observations. Cited with permission.

proteins have been identified, and even fewer have had their function defined. To fully utilize this technique in pharmacology, affected proteins must be identified by name and function, and studies such as ours need to be extended to other cell types and drug classes. The power of this technique will increase greatly when libraries of drug-induced changes are developed. Eventually, a 2-D gel "fingerprint" may suffice to directly derive mechanistic information.

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